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Request for grant of a patent

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1.	Your Reference	**RMT/PG4082**
2.	Patent application number *(The Patent office will fill in this part)*	~~21 JUL 2000~~ **0017990.3**
3.	Full name, address and postcode of the or of each applicant *(underline all surnames)*	**GLAXO GROUP LIMITED** **GLAXO WELLCOME HOUSE** **BERKELEY AVENUE** **GREENFORD** **MIDDLESEX** **UB6 0NN** **GB**
	Patents ADP number *(if you know it)*	07358700 3
	If the applicant is a corporate body, give the country/state of its corporation	**GB**
4	Title of the invention	**PAPILLOMA VIRUS SEQUENCES**
5	Name of your agent *(if you know one)*	**RACHEL M. THORNLEY** **(SEE CONTINUATION SHEET)**
	"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	**GLAXO WELLCOME PLC** **GLAXO WELLCOME HOUSE, BERKELEY AVENUE** **GREENFORD, MIDDLESEX** **UB6 ONN, GB**
	Patents ADP number *(if you know it)*	7062540001

6. If you are declaring priority from one or more earlier patent applications, give the country and date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number	Country	Priority application number *(if you know it)*	Date of Filing (day / month / year)

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application	Number of earlier application	Date of filing (day / month / year)

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a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
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Description	25
Claim(s)	4
Abstract	1
Drawing(s)	11

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Priority Documents	
Translations of priority documents	
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patent Form 9/77)*	
Request for substantive examination *(Patent Form 10/77)*	
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11. **I/We request the grant of a patent on the basis of this application**

21 July 2000

Signature **RACHEL M. THORNLEY** **Date 21st July 2000**

AGENT FOR THE APPLICANTS

12. Name and daytime telephone number of person to contact in the United Kingdom

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Papilloma Virus Sequences

The present invention relates to methods and compositions useful in the treatment and prevention of human papilloma virus infections and the symptoms and diseases associated therewith.

Papilloma virus infections have been observed in a variety of species, including sheep, dogs, rabbits, monkeys, cattle and humans. Human papilloma viruses (HPV) have been classified into more than 80 types [Epidemiology and Biology of Cervical Cancer Seminars in Surgical Oncology 1999 16:203-211. Wolfgang MJ, Schoell MD, Janicek MF and Mirhashemi R.] , some of which are further divided into sub-types (e.g. type 6a and 6b), based on the extent of DNA sequence homology. Papilloma viruses generally infect epithelia, but the different HPV types cause distinct diseases. For example, types 1-4, 7, 10 and 26-29 cause benign warts, types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 are associated with cervical cancers and types 6 and 11 are implicated in genital warts (non-malignant condylomata of the genital tract).

HPV has proven difficult to grow in tissue culture, so there is no traditional live or attenuated viral vaccine. Development of an HPV vaccine has also been slowed by the lack of a suitable animal model in which the human virus can be studied. This is because the viruses are highly species specific, so it is very difficult to infect an animal with a papilloma virus from a host of a different species, as would be required for safety testing before a vaccine was first tried in humans.

Papilloma viruses have a DNA genome which encodes "early" and "late" genes designated E1 to E7, L1 and L2. The early gene sequences have been shown to have functions relating to viral DNA replication and transcription, evasion of host immunity, and alteration of the normal host cell cycle and other processes. For example the E1 protein is an ATP-dependent DNA helicase and is involved in initiation of the viral DNA replication process whilst E2 is a regulatory protein controlling both viral gene expression and DNA replication. Through its ability to bind to both E1 and the viral origin of replication, E2 brings about a local concentration of E1 at the origin, thus stimulating the initiation of viral DNA replication. The E4 protein appears to have a number of poorly defined

functions but amongst these may be binding to the host cell cytoskeleton, whilst E5 appears to delay acidification of endosomes resulting in increased expression of EGF receptor at the cell surface and both E6 and E7 are known to bind cell proteins p53 and pRB respectively. The E6 and E7 proteins form HPV types associated with cervical cancer are known oncogenes. L1 and L2 encode the two viral structural (capsid) proteins.

Historically, vaccines have been seen as a way to prevent infection by a pathogen, priming the immune system to recognise the pathogen and neutralise it should an infection occur. The vaccine includes one or more antigens from the pathogen, commonly the entire organism, either killed or in a weakened (attenuated) form, or selected antigenic peptides from the organism. When the immune system is exposed to the antigen(s), cells are generated which retain an immunological "memory" of it for the lifetime of the individual. Subsequent exposure to the same antigen (e.g. upon infection by the pathogen) stimulates a specific immune response which results in elimination or inactivation of the infectious agent.

There are two arms to the immune response: a humoral (antibody) response and a cell-mediated response. Protein antigens derived from pathogens that replicate intracellularly (viruses and some bacteria) are processed within the infected host cell releasing short peptides which are subsequently displayed on the infected cell surface in association with class I major histocompatability (MHC I) molecules. When this associated complex of MHC I and peptide is contacted by antigen-specific CD8+ T-cells the T-cell is activated, acquiring cytotoxic activity. These cytotoxic T-cells (CTLs) can lyse infected host cells, so limiting the replication and spread of the infecting pathogen. Another important arm of the immune response is controlled by CD4+ T-cells. When antigen derived from pathogens is released into the extracellular milieu they may be taken up by specialised antigen-presenting cells (APCs) and displayed upon the surface of these cells in association with MHC II molecules. Recognition of antigen in this complex stimulates CD4+ T-cells to secrete soluble factors (cytokines) which regulate the effector mechanisms of other T-cells. Antibody is produced by B-cells. Binding of antigen to secreted antibody may neutralise the infectivity of a pathogen and binding of antigen to membrane-bound antibody on the surface of

B-cells stimulates division of the B-cell so amplifying the B-cell response. In general, good antibody responses are required to control bacterial infections and both antibody and cell-mediated immune responses (CD8+ and CD4+) are required to control infections by viruses.

It is believed that it may be possible to harness the immune system by vaccination, even after infection by a pathogen, to control or resolve the infection by inactivation or elimination of the pathogen. Such "therapeutic" vaccines would require a cell-mediated response to be effective, and would ideally invoke both humoral and cell-mediated immune responses.

It has been demonstrated (Benvenisty, N and Reshaf, L. PNAS 83 9551-9555) that inoculation of mice with calcium phosphate precipitated DNA results in expression of the peptides encoded by the DNA. Subsequently, intramuscular injection into mice of plasmid DNA which had not been precipitated was shown to result in uptake of the DNA into the muscle cells and expression of the encoded protein. Because expression of the DNA results in production of the encoded pathogen proteins within the host's cells, as in a natural infection, this mechanism can stimulate the cell-mediated immune response required for therapeutic vaccination. DNA vaccines are described in WO90/11092 (Vical, Inc.).

DNA vaccination may be delivered by mechanisms other than intra-muscular injection. For example, delivery into the skin takes advantage of the fact that immune mechanisms are highly active in tissues that are barriers to infection such as skin and mucous membranes. Delivery into skin could be via injection, via jet injector (which forces a liquid into the skin under pressure) or via particle bombardment, in which the DNA may be coated onto particles of sufficient density to penetrate the epithelium (US Patent No. 5371015). Projection of these particles into the skin results in direct transfection of both epidermal cells and epidermal Langerhan cells. Langerhan cells are antigen presenting cells (APC) which take up the DNA, express the encoded peptides, and process these for display on cell surface MHC proteins. Transfected Langerhan cells migrate to the lymph nodes where they present the displayed antigen fragments to lymphocytes, invoking an immune response. Very small amounts of DNA

(0.5-1 μg) are required to induce an immune response via particle delivery into skin and this contrasts with the milligram quantities of DNA known to be required to generate immune responses subsequent to direct intramuscular injection.

It has been reported, for example in studies using virus like particles formed from the L1 and L2 capsid proteins or using these proteins alone (1), that HPV is poorly immunogenic. Furthermore, HPV genes have proven difficult to express in human or other mammalian cells, leading difficulties in developing protein subunit vaccines. Monocystronic E1 has proven particularly resistant to expression from heterologous promoters in mammalian cells (J.Virology 1999 73, 3062-3070. Remm M, Remm A and Mart Ustav. Human papilloma virus type 18 E1 is translated from polycistronic mRNA by a discontinuous scanning mechanism). Expression of E1 is most often detected using *in vitro* DNA replication of an HPV origin containing plasmid as a surrogate (Lu, JZJ, Sun et al J.Virol 1993 67, 7131-7139 and Del Vecchio AM et al J.Virol 1992 66, 5949-5958).

The DNA code has 4 letters (ATC and G) and uses these to spell three letter "codons" which represent the amino acids the proteins encoded in an organism's genes. The linear sequence of codons along the DNA molecule is translated into the linear sequence of amino acids in the protein(s) encoded by those genes. The code is highly degenerate, with 61 codons coding for the 20 natural amino acids and 3 codons representing "stop" signals. Thus, most amino acids are coded for by more than one codon - in fact several are coded for by four or more different codons.

Where more than one codon is available to code for a given amino acid, it has been observed that the codon usage patterns of organisms are highly non-random. Different species show a different bias in their codon selection and, furthermore, utilization of codons may be markedly different in a single species between genes which are expressed at high and low levels. This bias is different in viruses, plants, bacteria and mammalian cells, and some species show a stronger bias away from a random codon selection than others. For example, humans and other mammals are less strongly biased than certain bacteria or viruses. For these reasons, there is a significant probability that a mammalian

gene expressed in E.coli or a viral gene expressed in mammalian cells will have an inappropriate distribution of codons for efficient expression. However, a gene with a codon usage pattern suitable for E.coli expression may also be efficiently expressed in humans. It is believed that the presence in a heterologous DNA sequence of clusters of codons which are rarely observed in the host in which expression is to occur is predictive of low heterologous expression levels.

There are several examples where changing codons from those which are rare in the host to those which are host-preferred ("codon optimisation") has enhanced heterologous expression levels, for example the BPV (bovine papilloma virus) late genes L1 and L2 have been codon optimised for mammalian codon usage patterns and this has been shown to give increased expression levels over the wild-type HPV sequences in mammalian (Cos-1) cell culture (Zhou et. al. 1999 J. Virol. Vol. 73 No. 6 pp 4972-4982). In this work, every BPV codon which occurred more than twice as frequently in BPV than in mammals (ration of usage >2), and most codons with a usage ratio of >1.5 were conservatively replaced by the preferentially used mammalian codon. In WO97/31115, WO97/48370 and WO98/34640 (Merck & Co., Inc.) codon optimisation of HIV genes or segments thereof has been shown to result in increased protein expression and improved immunogenicity when the codon optimised sequences are used as DNA vaccines in the host mammal for which the optimisation was tailored. In this work, the sequences consist entirely of optimised codons (except where this would introduce an undesired restriction site, intron splice site etc.) because each viral codon is conservatively replaced with the optimal codon for the intended host.

According to a first aspect, the present invention provides a polynucleotide sequence which encodes an HPV amino acid sequence, wherein the codon usage pattern of the polynucleotide sequence resembles that of highly expressed mammalian genes. Desirably the codon usage pattern of the polynucleotide sequence is typical of highly expressed human genes. Ideally, the codon usage pattern of the polynucleotide sequence also resembles that of highly expressed E.coli genes. The polynucleotide sequence may be a DNA sequence, for example a double stranded DNA sequence. Preferably the polynucleotide sequence encodes a HPV polypeptide of HPV type 6, 11, 16, 18,

33 or 45, most preferably type 11, sub-type 6a or sub-type 6b. In certain embodiments the encoded amino acid sequence is a wild-type HPV amino acid sequence. In alternative embodiments, the encoded amino acid sequence is a mutated HPV amino acid sequence comprising the wild-type sequence with amino acid changes, for example amino acid point mutations, sufficient to reduce or inactivate one or more of the natural biological functions of the polypeptide. The mutated amino acid sequence will desirably retain the immunogenicity of the wild-type polypeptide.

The encoded HPV polypeptide may be an early gene product such as E1, E2 or E7, or a fragment thereof, or may be a late gene product such as L1 or L2, or a fragment thereof. Preferably, the encoded HPV polypeptide is the whole or a part of an early gene product, most preferably E1 or E2. In one particular embodiment, the polynucleotide sequence encodes the wild-type HPV E1 polypeptide of sub-type 6b as set out in Fig. 1. In alternative embodiments, the polynucleotide sequence may encode one or more of the mutated HPV6b E1 amino acid sequence as set out in Fig. 2, the wild-type E2 amino acid sequence of HPV type 11 (Fig. 3) or of sub-type 6b (in Fig. 3), the mutated HPV6b E2 amino acid sequence (Fig. 4) and the mutated HPV11 E2 sequence as set out in Fig. 4.

According to the present invention, the codon usage pattern of the polynucleotide will preferably exclude codons with an RSCU value of less than 0.2 in highly expressed genes of the target organism. A relative synonymous codon usage (RSCU) value is the observed number of codons divided by the number expected if all codons for that amino acid were used equally frequently. A polynucleotide of the present invention will generally have a codon usage coefficient for highly expressed human genes of greater than 0.3, preferably greater than 0.4, most preferably greater than 0.5. desirably the polynucleotide will also have a codon usage coefficient for highly expressed E.coli genes of greater than 0.5, preferably greater than 0.6, most preferably greater than 0.7.

In one embodiment, the present invention provides a polynucleotide sequence as set out in Fig. 5 or Fig. 6, or a fragment or variant thereof which maintains the codon usage pattern thereof. In a further embodiment, the present invention

provides a polynucleotide sequence complementary to the sequence set out in Fig. 5 or Fig. 6.

According to a second aspect of the invention, an expression vector is provided which comprises and is capable of directing the expression of a polynucleotide sequence according to the first aspect of the invention, encoding an HPV amino acid sequence wherein the codon usage pattern of the polynucleotide sequence is typical of highly expressed mammalian genes, preferably highly expressed human genes. The vector may be suitable for driving expression of heterologous DNA in bacterial insect or mammalian cells, particularly human cells. In one embodiment, the expression vector is p7313PLc (Fig.7).

According to a third aspect of the invention, a host cell comprising a polynucleotide sequence according to the first aspect of the invention, or an expression vector according the second aspect, is provided. The host cell may be bacterial, e.g. E.coli, mammalian, e.g. human or may be an insect cell. Mammalian cells comprising a vector according to the present invention may be cultured cells transfected in vitro or may be transfected in vivo by administration of the vector to the mammal.

In a fourth aspect, the present invention provides a vaccine composition comprising a polynucleotide sequence according to the first aspect of the invention. Preferably the vaccine composition comprises a DNA vector according to the second aspect of the present invention. In preferred embodiments the vaccine composition comprises a plurality of particles, preferably gold particles, coated with DNA comprising a vector encoding a polynucleotide sequence which encodes an HPV amino acid sequence, wherein the codon usage pattern of the polynucleotide sequence is typical of highly expressed mammalian genes, particularly human genes. In alternative embodiments, the vaccine composition comprises a pharmaceutically acceptable excipient and a DNA vector according to the second aspect of the present invention. The vaccine composition may also include an adjuvant.

In a further aspect, the present invention provides a method of making a vaccine composition including the step of altering the codon usage pattern of a wild-type

HPV nucleotide sequence to produce a sequence having a codon usage pattern typical of highly expressed mammalian genes and encoding a wild-type HPV amino acid sequence or a mutated HPV amino acid sequence comprising the wild-type sequence with amino acid changes sufficient to inactivate one or more of the natural functions of the polypeptide.

Also provided are the use of a polynucleotide according to the first aspect, or of a vector according to a second aspect of the invention, in the treatment or prophylaxis of an HPV infection, preferably an infection of HPV type 6, 11, 16 or 18. The invention also provides the use of a polynucleotide according to the first aspect, a vector according to the second aspect of the invention or a vaccine according to the fourth aspect of the invention, in the treatment or prophylaxis of cutaneous (skin) warts, genital warts, atypical squamous cells of undetermined significance (ASCUS), cervical dysplasia, cervical intraepithelial neoplasia (CIN) or cervical cancer. Accordingly, the present invention also provides the use of a polynucleotide according to the first aspect, or of a vector according to the second aspect of the invention in making a vaccine for the treatment or prophylaxis of an HPV infection or any symptoms or disease associated therewith.

The present invention also provides methods of treating or preventing HPV infections or any symptoms or diseases associated therewith comprising administering an effective amount of a polynucleotide according to the first aspect, a vector according to the second aspect or a vaccine according to the fourth aspect of the invention. Administration of a vaccine may take the form of one or more individual doses, for example in a "prime-boost" regime. In certain cases the "prime" vaccination may be via particle mediated DNA delivery of a polynucleotide according to the present invention, preferably incorporated into a plasmid-derived vector and the "boost" by administration of a recombinant viral vector comprising the same polynucleotide sequence.

Throughout the present specification and the accompanying claims the words "comprise" and "include" and variations such as "comprises", "comprising", "includes" and "including" are to be interpreted inclusively. That is, these words

are intended to convey the possible inclusion of other elements or integers not specifically recited, where the context allows.

The term "variant" refers to a polynucleotide which encodes the same amino acid sequence as another polynucleotide of the present invention but which, through the redundancy of the genetic code, has a different nucleotide sequence whilst maintaining the same codon usage pattern, for example having the same codon usage coefficient or a codon usage coefficient within 0.1, preferably within 0.05 of that of the other polynucleotide.

The term "codon usage pattern" refers to the average frequencies for all codons in the nucleotide sequence, gene or class of genes under discussion (e.g. highly expressed mammalian genes). Codon usage patterns for mammals, including humans can be found in the literature (see e.g. Nakamura et.al. Nucleic Acids Research 1996, **24**:214-215).

In the polynucleotides of the present invention, the codon usage pattern is altered from that typical of human papilloma viruses to more closely represent the codon bias of the target organism, e.g. E.coli or a mammal, especially a human. The "codon usage coefficient" is a measure of how closely the codon pattern of a given polynucleotide sequence resembles that of a target species. Codon frequencies can be derived from literature sources for the highly expressed genes of many species (see e.g. Nakamura et.al. Nucleic Acids Research 1996, **24**:214-215). The codon frequencies for each of the 61 codons (expressed as the number of occurrences occurrence per 1000 codons of the selected class of genes) are normalised for each of the twenty natural amino acids, so that the value for the most frequently used codon for each amino acid is set to 1 and the frequencies for the less common codons are scaled to lie between zero and 1. Thus each of the 61 codons is assigned a value of 1 or lower for the highly expressed genes of the target species. In order to calculate a

codon usage coefficient for a specific polynucleotide, relative to the highly expressed genes of that species, the scaled value for each codon of the specific polynucleotide are noted and the geometric mean of all these values is taken (by dividing the sum of the natural logs of these values by the total number of codons and take the anti-log). The coefficient will have a value between zero and 1 and the higher the coefficient the more codons in the polynucleotide are frequently used codons. If a polynucleotide sequence has a codon usage coefficient of 1, all of the codons are "most frequent" codons for highly expressed genes of the target species.

Shorter polynucleotide sequences are within the scope of the invention. For example, a polynucleotide of the invention may encode a fragment of a HPV protein. A polynucleotide which encodes a fragment of at least 8, for example 1-10 amino acids or up to 20, 50, 60, 70, 80, 100, 150 or 200 amino acids in length is considered to fall within the scope of the invention as long as the polynucleotide has a codon usage pattern which resembles that of a highly expressed mammalian gene and the encoded oligo or polypeptide demonstrates HPV antigenicity. In particular, but not exclusively, this aspect of the invention encompasses the situation when the polynucleotide encodes a fragment of a complete HPV protein sequence and may represent one or more discrete epitopes of that protein.

The polynucleotides of the present invention show higher expression in E.coli and mammalian cells than corresponding wild-type sequences. Whilst not wishing to be bound by any theory, this is believed to be for at least two reasons. Firstly, having a codon usage pattern closer to that of the host cell, the sequences are more easily processed by the cell translation machinery. Secondly, as up to 30% of the nucleotide sequence (or more) is different from the wild-type sequence, sites which interfere with translation (such as protein binding sites) will have been removed or altered.

In some embodiments, polynucleotides according tot he present invention show codon usage patterns which resemble those of E.coli and mammalian (e.g. human) genes. This is particularly advantageous where a sequence is to be used in vaccination of a mammal and in generation of significant amounts of the antigen protein in vitro using E.coli cells (e.g. for use in assays, such as immunoassays to judge the levels of expression in mammalian or human tissues).

As discussed above, the present invention includes expression vectors that comprise the nucleotide sequences of the invention. Such expression vectors are routinely constructed in the art of molecular biology and may for example involve the use of plasmid DNA and appropriate initiators, promoters, enhancers and other elements, such as for example polyadenylation signals which may be necessary, and which are positioned in the correct orientation, in order to allow for protein expression. Other suitable vectors would be apparent to persons skilled in the art. By way of further example in this regard we refer to Sambrook *et al*. Molecular Cloning: a Laboratory Manual. 2nd Edition. CSH Laboratory Press. (1989).

Preferably, a polynucleotide of the invention or for use in the invention in a vector is operably linked to a control sequence which is capable of providing for the expression of the coding sequence by the host cell, i.e. the vector is an expression vector. The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A regulatory sequence, such as a promoter, "operably linked" to a coding sequence is positioned in such a way that expression of the coding sequence is achieved under conditions compatible with the regulatory sequence.

The vectors may be for example, plasmid, artificial chromosome, virus or phage vectors provided with a origin of replication, optionally a promoter for the expression of the said polynucleotide and optionally a regulator of the promoter. The vectors may contain one or more selectable marker genes, for example an ampicillin or kanomycin resistance gene in the case of a bacterial plasmid or a resistance gene for a fungal vector. Vectors may be used *in vitro*, for example for the production of DNA or RNA or used to transfect or transform a host cell, for example, a mammalian host cell. The vectors may also be adapted to be used *in vivo*, for example in a method of DNA vaccination or of gene therapy.

Promoters and other expression regulation signals may be selected to be compatible with the host cell for which expression is designed. For example, mammalian promoters include the metallothionein promoter, which can be induced in response to heavy metals such as cadmium, and the β-actin promoter. Viral promoters such as the SV40 large T antigen promoter, human cytomegalovirus (CMV) immediate early (IE) promoter, rous sarcoma virus LTR promoter, adenovirus promoter), or a HPV promoter, particularly the HPV upstream regulatory region (URR) may also be used. All these promoters are readily available in the art.

Examples of suitable viral vectors include herpes simplex viral vectors, vaccinia or alpha-virus vectors and retroviruses, including lentiviruses, adenoviruses and adeno-associated viruses. Gene transfer techniques using these viruses are known to those skilled in the art. Retrovirus vectors for example may be used to stably integrate the polynucleotide of the invention into the host genome, although such recombination is not preferred. Replication-defective adenovirus vectors by contrast remain episomal and therefore allow transient expression. Vectors capable of driving expression in insect cells (for example baculovirus vectors), in human cells or in bacteria may be employed in order to produce

quantities of the HPV protein encoded by the polynucleotides of the present invention, for example for use as subunit vaccines or in immunoassays.

The polynucleotides according to the invention have utility in the production, by expression, of the proteins according to the invention, which expression may take place *in vitro, in vivo* or *ex vivo.* The nucleotides may therefore be involved in recombinant protein synthesis, for example to increase yields, or indeed may find use as therapeutic agents in their own right, utilised in DNA vaccination techniques. Where the polynucleotides of the present invention are used in the production of the encoded proteins *in vitro* or *ex vivo*, cells, for example in cell culture, will be modified to include the polynucleotide to be expressed. Such cells include transient, or preferably stable mammalian cell lines. Particular examples of cells which may be modified by insertion of vectors encoding for a polypeptide according to the invention include mammalian HEK293T, CHO, HeLa and COS cells. Preferably the cell line selected will be one which is not only stable, but also allows for mature glycosylation and cell surface expression of a polypeptide. Expression may be achieved in transformed oocytes. A polypeptide of the invention may be expressed in cells of a transgenic non-human animal, preferably a mouse. A transgenic non-human animal expressing a polypeptide of the invention is included within the scope of the invention.

Where the polynucleotides of the present invention find use as therapeutic agents, e.g. in DNA vaccination, the nucleic acid will be administered to the mammal e.g. human to be vaccinated. The nucleic acid, such as RNA or DNA, preferably DNA, is provided in the form of a vector, such as those described above, which may be expressed in the cells of the mammal. The polynucleotides may be administered by any available technique. For example, the nucleic acid may be introduced by needle injection, preferably intradermally, subcutaneously or intramuscularly. Alternatively, the nucleic acid may be delivered directly across the skin using a nucleic acid delivery device such as particle-mediated

DNA delivery (PMDD).In this method, inert particles (such as gold beads) are coated with a nucleic acid, and are accelerated at speeds sufficient to enable them to penetrate a surface of a recipient (e.g. skin), for example by means of discharge under high pressure from a projecting device. (Particles coated with a nucleic acid molecule of the present invention are within the scope of the present invention, as are devices loaded with such particles).

Suitable techniques for introducing the naked polynucleotide or vector into a patient include topical application with an appropriate vehicle. The nucleic acid may be administered topically to the skin, or to mucosal surfaces for example by intranasal, oral, intravaginal or intrarectal administration. The naked polynucleotide or vector may be present together with a pharmaceutically acceptable excipient, such as phosphate buffered saline (PBS). DNA uptake may be further facilitated by addition of facilitating agents such as bupivacaine to the composition. Other methods of administering the nucleic acid directly to a recipient include ultrasound, electrical stimulation, electroporation and microseeding which is described in US-5,697,901.

Uptake of nucleic acid constructs may be enhanced by several known transfection techniques, for example those including the use of transfection agents. Examples of these agents includes cationic agents, for example, calcium phosphate and DEAE-Dextran and lipofectants, for example, lipofectam and transfectam. The dosage of the nucleic acid to be administered can be altered. Typically the nucleic acid is administered in an amount in the range of 1pg to 1mg, preferably to 1pg to 10μg nucleic acid for particle mediated gene delivery and 10μg to 1mg for other routes.

A nucleic acid sequence of the present invention may also be administered by means of specialised delivery vectors useful in gene therapy. Gene therapy approaches are discussed for example by Verme *et al*, Nature 1997, **389**:239-242. Both viral and non-viral systems can be used. Viral based systems include

retroviral, lentiviral, adenoviral, adeno-associated viral, herpes viral, Canarypox and vaccinia-viral based systems. Non-viral based systems include direct administration of nucleic acids and liposome-based systems.

A nucleic acid sequence of the present invention may also be administered by means of transformed cells. Such cells include cells harvested from a subject. The naked polynucleotide or vector of the present invention can be introduced into such cells *in vitro* and the transformed cells can later be returned to the subject. The polynucleotide of the invention may integrate into nucleic acid already present in a cell by homologous recombination events. A transformed cell may, if desired, be grown up *in vitro* and one or more of the resultant cells may be used in the present invention. Cells can be provided at an appropriate site in a patient by known surgical or microsurgical techniques (e.g. grafting, micro-injection, etc.)

The vaccine compositions of the present invention may include adjuvant compounds which may serve to increase the immune response induced by the protein which is encoded by the plasmid DNA. Alteration of the codon bias to suit the vaccinated species is proposed herein as a means of increasing expression and thereby boosting the immune response, but an adjuvant may never-the-less be desirable because, while DNA vaccines tend to work well in mice models, there is evidence of a somewhat weaker potency in larger species such as non-human primates which is thought to be predictive of the likely potency in humans.

The following Examples serve to further illustrate the invention, with reference to the accompanying drawings, in which:

Figure 1 shows the consensus wild-type nucleotide sequences of E1 from HPV types 11, 6a and 6b, derived from Genbank **[is this where they came from?]**;

Figure 2 shows the amino acid sequence of HPV6b E1 from Fig. 1 with point mutations to remove biological activity;

Figure 3 shows the consensus wild-type nucleotide sequences of E2 from HPV types 11, 6a and 6b, derived from Genbank **[is this where they came from?]**;

Figure 4 shows the amino acid sequences of HPV6b and HPV11 E2 from Fig. 3 with point mutations to remove biological activity;

Figure 5 shows a nucleotide sequence, having a codon usage pattern resembling that of a highly expressed human gene, encoding the amino acid sequence of HPV6b E1 from Fig. 1;

Figure 6 shows a nucleotide sequence, having a codon usage pattern resembling that of a highly expressed human gene, encoding the amino acid sequence of HPV6b E2 from Fig. 3;

Figure 7 shows DNA vector p7313-PLc;

Figure 8 shows cell lysate samples from Example 3 run on an acrylamide gel and stained to show antibody binding to expressed E1 protein;

Figure 9 shows cellular responses to antigen challenge after immunisation of mice with a polynucleotide according to the invention.

Example 1 - Codon Optimisation of HPV6bE1

The wild-type consensus amino acid sequence of HPV6b E1, obtained from Genebank, is set out in Fig.1(bottom sequence). This figure shows the high level of homology for this protein between the HPV virus types 11, 6a and 6b. Similarly, Figure 3 sets out the wild-type consensus amino acid sequences for the E2 protein of HPV11, 6a and 6b. It is expected that a vaccine using HPV6b sequences will cross-react to provide a prophylactic or therapeutic immune response against all three viral types.

The codon usage of the HPV6b E1 sequence was compared to that of highly expressed human and E.coli genes and found to have a low codon usage coefficient. Simply using the most abundant codon for each amino acid residue

would result in an equally skewed codon usage pattern. Consequently, the codons were assigned using a statistical method to give synthetic gene having a codon frequency closer as possible to that found naturally in highly expressed E.coli and human genes.

The codons in the synthetic gene were assigned using a Visual Basic program called Calcgene, written by R. S. Hale and G Thompson (Protein Expression and Purification Vol. 12 pp.185-188 (1998)). For each amino acid residue in the original sequence, a codon was assigned based on the probability of it appearing in highly expressed E.coli genes. Details of the program, which works under Microsoft Windows 3.1, can be obtained from the authors. Because the program applies a statistical method to assign codons to the synthetic gene, not all resulting codons are the most frequently used in the target organism. Rather, the proportion of frequently and infrequently used codons of the target organism is reflected in the synthetic sequence by assigning codons in the correct proportions. However, as there is no hard-and-fast rule assigning a particular codon to a particular position in the sequence, each time it is run the program will produce a different synthetic gene - although each will have the same codon usage pattern and each will encode the same amino acid sequence. If the program is run several times for a given amino acid sequence and a given target organism, several nucleotide sequences will be produced which may differ in the number, type and position of restriction sites, intron splice signals etc., some of which may be undesirable. The skilled artisan will be able to select an appropriate sequence for use in expression of the polypeptide on the basis of these features.

Furthermore, since the codons are randomly assigned on a statistical basis, it is possible (although perhaps unlikely) that two or more codons which are relatively rarely used in the target organism might be clustered in close proximity. It is believed that such clusters may upset the machinery of translation and result in particularly low expression rates, so the algorithm for choosing the codons in the optimized gene excluded any codons with an RSCU value of less than 0.2 for highly expressed genes in order to prevent any rare codon clusters being fortuitously selected. The distribution of the remaining codons were then allocated according to the frequencies for highly expressed E.coli to give an

overall distribution within the synthetic gene that was typical such genes (coefficient = 0.85) and also for highly expressed human genes (coefficient = 0.50). The wild type and codon optimised nucleotide sequences for HPV6b E1 are shown in Fig. 5.

The same process was used to obtain a nucleotide sequence for HPV11 E2, but allocating codons according to the codon frequency pattern of highly expressed human genes. The wild type and codon optimised nucleotide sequences for HPV6b E1 are shown in Fig. 6.

Point mutations were also introduced into the E1 gene (K83G, R84G and G482D) and E2 gene (K111A) to ensure that these proteins had lost all of their known biochemical activities. The amino acid sequences of the mutated genes are shown (aligned with the wild-type sequence) in Figs. 2 and 4 **[something is wrong with the aa seq of E1 derived from Fig1 - position 482 is not G]**

Example 2 - Construction of the Codon Optimised HPV6b E1 Polynucleotide Sequence

Gene Design:

Using the optimisation software discussed above, overlapping 40mer oligonucleotides were calculated from the optimised sequence. The terminal oligonucleotides containing the restriction sites were 60mers. The oligonucleotides were ordered from Life Technologies Ltd at 50nmole concentration, deprotected and non-phosphorylated.

Oligonucleotide assembly:

Each oligonucleotide was dissolved in double distilled water to a final concentration of 100 micromolar (μM) and an equal mixture was prepared of all 96 oligonucleotides at 100 μM. The synthesis was set up as follows using Pwo polymerase from Roche Boehringer (Cat No. 1 644 955).

Double distilled water	86µl
Pwo 10X buffer	10µl
dNTP mix	1µl (equal mix of 100mM dNTPs)
oligo mix	1µl (equal mix of 100µM oligos)
Pwo polymerase	2µl.

A Polymerase Chain Reaction (PCR) was carried out on the above reaction mix on a Trio Thermoblock (Biometra)using the following conditions:

1. 40°C 2 min
2. 72°C 10 sec
3. 94°C 15 sec
4. 40°C 30 sec
5. 72°C 20 sec + 2secs per cycle
6. 4°C ∞

Cycle repeated 25 times between steps 3 and 5.

After completion of 25 cycles, a 10µl aliquot was removed from each tube and run on a 0.8% Tris Acetate (TAE) agarose gel and observed under long wave UV light. The expected size of the synthesised E1 DNA should be 1.958 kilo base pairs (kb).

Gene Recovery:

The synthetic gene was recovered by PCR using polymerase using the two terminal oligos which contained a Not 1 restriction site on the N terminal of the synthetic oligo and a Bam H1 site at the C terminal synthetic oligo.

Double distilled water	65µl
Pwo 10X buffer	10µl
dNTP mix	1µl (equal mix of 100mM dNTPs)
assembly mix	20µl (from previous PCR)
N terminal oligo	1µl (100µM e1s: u1)
C terminal oligo	1µl (100µM e1s: l49)
Pwo polymerase	2µl.

1. 94°C 45 sec
2. 72°C 2 min + 1 min per 500bp
3. 72°C 10 min
4. 4°C ∞

Cycle repeated 25 times between steps 2 and 1.

The PCR product was then cleaned up using a QIAquick PCR purification kit (Qiagen Cat No. 28104) and DNA resuspended in a total of 50µl of elution buffer. A 10µl aliquot was digested with Not 1 and BamH1 restriction enzymes (from Life Technologies Ltd, 3 Fountain Drive, Inchinnan Business Park, Paisley, Scotland) for 2 hours at 37°C. This digest was gel purified on 0.8% TAE agarose gel. A 2kb DNA product was excised and extracted using a QIAquick Gel extraction Kit (Qiagen Cat No. 28704). The final product was eluted in a total of 50µl of elution buffer.

This Not1-BamH1 fragment of DNA was ligated into pCIN4 vector or pcDNA3.1 also digested with Not1-BamH1, gel extracted using a QIAquick Gel extraction Kit (Qiagen Cat No. 28704) and ligated into vectors using T4 DNA Ligase (Promega UK Ltd Delta House, Chilworth Research Centre, Southampton, UK)for 2 hours at room temperature. A 2 µl aliquot was transformed using JM109 chemically competent E.coli cells from Promega (Cat no: P9751). The transformation was plated out on 2X Luria Broth (LB) agar plates with 50µg/ml Ampicillin in the agar. The plates were inverted and incubated overnight at 37°C. Any resulting colonies were individually picked in 5 ml of LB media with 50µg/ml Ampicillin selection. These were grown shaking at 37°C for 5 hours and mini-prepped using Qiagen DNA miniprep spin kit (Qiagen, Cat No. 27104). A 5µl aliquot was digested with Nco1-BamH1 and Nco1-EcoR1 restriction enzymes to check the clones.

5 correct clones with 2kb fragments were sequenced and clone 17 had 3 point mutations. The point mutations were corrected by ligation of fragments with correct sequences from several different clones and cloned into vector p7313-PLc. The vector (Fig. 7) has a minimal CMV promoter and intron (WO00/23592 - Powderject Vaccines Inc.).

The resulting clones were checked for correct insert, sequenced for comparison with the expected sequence and scaled up via a Qiagen DNA maxi-prep kit. At the same time a Not1-BamH1 fragment derived from the wild-type consensus sequence of HPV6bE1 was also cloned into the p7313-PLc vector, sequenced and used for comparison with the codon optimised version.

Composition of plasmid p7313-PLc

The plasmid was constructed by replacing the beta-lactamase gene containing Eam1105I - Pst1 fragment of pUC19 with an EcoRI fragment of pUC4K (Amersham-Pharmacia) containing the Kanomycin resistance gene, following blunt ending of both fragments using T4 DNA polymerase. The human Cytomegalovirus IE1 promoter /enhancer, Intron A, was derived from plasmid JW4303 obtained from Dr Harriet Robinson, University of Massachusetts, and inserted into the Sal1 site of pUC19 as a XhoI -Sal1 fragment, incorporating the bovine growth hormone polyadenylation signal. deletion of the 5' SalI-BanI fragment from the promoter generated the minimal promoter used in the vector. HBV Surface antigen 3'UTR was derived from Hepatitis B Virus, serotype adw, in the vector pAM6 (Moriarty et al., Proc.Natl.Acad.Sci. USA, 78, 2606-2610, 1981). pAM6 (pBR322 based vector) was obtained from the American Type Culture Collection, catalogue number ATCC 45020. The 3'UTR was inserted 5' to the polyadenylation signal as a 1.4kb BamHI fragment, blunt ended for insertion to remove the BamHI sites. In a series of steps (including digestion with *Bgl* II, Klenow polymerase treatment, digestion with *Bst*X I, digestion with *Nco* I, treatment with mung bean nuclease to remove overhang and further digestion with *Bst*X I), modifications were made to the region between the 3'untranslated enhancer region of the HBV S gene and bGHpA signal to remove all open reading frames of greater than 5 codons between the X gene promoter and the bGHpA signal. This resulted in deletion of sequence encoding the translatable portion of the X protein (9 amino acids) and the X gene start codon. However, the weak enhancer/promoter region of the X gene was retained because this region was found to enhance expression of HBsAg from the CMV promoter. The bovine growth hormone polyadenylation signal was substituted with the rabbit beta globin polyadenylation signal. The 5'non-coding and coding sequences of

the S antigen were excised and replaced with an oligonucleotide linker to provide multiple cloning sites as shown to produce plasmid p7313-PL.

```
Hind    -- - Not I - --        -EcoRV        --NdeI--        --BamHI
AGCTTGCGGCCGCTAGCGATATCGGTACCATATGTCGACGGATCC . . . .
. . . . ACGCCGGCGATCGCTATAGCCATGGTCTACAGCTGCCTAGGCCGG
                --NheI--        --KpnI--        -- SalI --        ΔNotI
```

The ColE1 cer sequence was obtained from a subclone from plasmid pDAH212 from David Hodgeson (Warwick University) and amplified by PCR using primers to place EcoRI restriction sites at the ends of the sequence. The cer sequence was then inserted into the EcoRI site of p7313-PL to produce plasmid p7313-PLc. The sequence of the amplified cer was verified against the Genbank entry M11411.

Example 3 - Expression in mammalian 293T cells

Mammalian 293T cells were grown at log phase at a final concentration of 2 $X10^5$ cells per 6 well Corning Costar™ (Corning Science Products, 10 The ValleyCentre, Gordon Road, High Wycombe, Bucks, UK)tissue culture plate overnight at 37°C in 5%CO_2. The following transfection mix was prepared and complexed for 25 minutes:

WRG7077 codon optimised HPV6bE1	2μg
Or WRG7077 wild-type HPV6bE1	2μg
Made up with sterile double distilled water	16μl
OPTI-mem™ (Gibco BRL, Paisley, Scotland)	8μl
Lipofectamine™ (GibcoBRL)	6μl.

Each cell monolayer in a well was washed carefully twice with OPTI-mem™. 800μl of OPTI-mem™ was added to each well. 200μl of OPTI-mem™ was added to each transfection mix, mixed and added gently to a cell monolayer. The plate was incubated for 5 hours at 37°C in 5% CO_2 after which the transfection mix and OPTI-mem™ were discarded. The cell monolayers were washed gently with cell growth medium twice and finally transfected cells were

incubated for 24 hours in Dulbecco's Modified Eagle Medium containing 10% foetal calf serum and 29.2mg/ml of L-glutamine at 37°C in 5% CO_2. The cells were scraped off into microtubes, washed twice with PBS, spun down and the cell pellet was resuspended in SDS Page Laemmli dye. The cell pellets were boiled and loaded onto a 10%SDS Page gel, electrophoresed in 1X Tris Glycine SDS buffer. After electrophoresis, the gel was blotted onto Nitrocellulose membrane (Amersham) and Western Blotted. The nitrocellulose membrane was blocked with 5% Marvel™ (Premier Beverages, Knighton, Adbaston, Stafford, UK) in PBS for 30 min at room temperature and washed twice with PBS and 0.1% Tween 20. A polyclonal antibody raised against the C terminal protein sequence of HPV6bE1 (protein sequence: CSSSLDIQDSEDEEDGSNSQAFR) in rabbits, was diluted in 5% Marvel™ in PBS and added to the nitrocellulose membrane. This was incubated at room temperature for 1 hour with gentle agitation. A polyclonal antibody against HPV11E1 was also used to check cross reactivity. The diluted antibody was removed and the membrane washed three times with PBS and 0.1% Tween 20. A secondary conjugate, Swine anti-rabbit horseradish peroxidase (HRP) (DAKO), was diluted 1:20000 in PBS and 0.1% Tween 20. This was added to the washed membrane and incubated with gentle agitation at room temperature for 1 hour. The membrane was then washed thoroughly with PBS and 0.1%Tween20. A Chemiluminescent HRP kit (Amersham) was used to detect the transferred proteins on the membrane.

Results:

The predicted size of a translated protein for E1 is ~70-80kDa. The results (Fig. 8) show a correct protein size expressed by p7313-PLc containing the codon optimised HPV6bE1 (lane 4). The size markers are in lane 5 and the positive control (purified E1 protein) was run in lane 6. Vector containing wild-type E1 is in lane 3, which shows that there was no detectable expression of E1 in human cells from the wild-type nucleotide sequence. Similarly no E1 is detected in lane 2 (empty vector) and lane 1 (untransfected cells). The approx 60kD band in lanes 1-4 is an unidentified cellular protein which cross-reacts with the anti-E1 antibody. The band is of roughly constant intensity across the lanes, showing that the loading of the samples was consistent.

Example 4 - Immunology - detection of cellular responses to HPV antigens

All reagents were obtained from Gibco BRL, Paisley, Scotland or Sigma, Poole, Dorset unless otherwise stated.

A. Immunisation protocol.

Female C57BL/6 mice were immunised with 1.0-2.0 μg DNA by PMDD and boosted with an identical dose 28 days later. Animals were sacrificed by cervical dislocation on day 42 and spleens removed for investigation of the cellular responses to HPV antigens.

B. Preparation of single cell suspension of splenocytes.

Spleens were "mashed" between ground glass slides (BDH), red blood cells lysed (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) and cells resuspended in complete RPMI. (RPMI-1640 medium supplemented with 10% foetal calf serum (FCS), 2mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 5 x 10^{-5} M 2-mercaptoethanol).

C. Infection of MC57 target cells.

Immunodominant epitopes derived from HPV antigens remain undefined therefore the detection of antigen specific responses *in vitro* relies on natural processing of whole antigen generated within target cells that have been transfected with cDNA encoding the whole protein(s). MC57 cells (K^b positive) were infected with recombinant vaccinia using a multiplicity of infection of 5 for 1 hour at 37°C. Excess virus was washed off and cells resuspended in complete RPMI containing 50 ng/ml recombinant human IL-2 (Glaxo Wellcome, Geneva).

D. ELISPOT.

ELISPOT plates (96 well, Millipore MAIP S 45 1 0) were coated with rat anti-mouse IFN gamma (Pharmingen 18181D) at 15 μg/ml in PBS overnight (4°C) prior to the addition of 4 x 10e5 splenocytes obtained from experimental groups. Antigen was presented by the addition of 1 x 10e4 recombinant vaccinia infected MC57 cells. Wild-type vaccinia was used as a negative control and influenza nucleoprotein (NP) (strainA/PR8/34) used as a negative control in this system. The assay was incubated overnight at 37°C(5% CO_2).

On day 2 of the assay, spot forming cells were detected using biotinylated rat anti-mouse IFN gamma (Pharmingen 18112D) at 1 μg /ml followed by streptavidin alkaline phosphatase conjugate (TCS biologicals SA 1008) at 1/1000 dilution in PBS. This was visualised using an alkaline phosphatase substrate kit (Biorad 170-6432) and quantified by image analysis. The results are shown in Fig. 9.

As can be seen from Fig. 9, a strong cellular response was seen from all three mice vaccinated with the E1 codon optimised sequence. No response was seen when these mice were challenged with wild-type vaccinia (WT), vaccinia carrying 'flu nucleoprotein (NP), or vaccinia carrying E1. Mice vaccinated with empty pVAC vector showed no response to any challenge, whilst the mouse vaccinated with flu nucleoprotein DNA showed a strong cellular response to challenge with vaccinia carrying NP, but to no other challenge.

The application of which this description and claims form part may be used as a basis for priority in respect of any subsequent application. The claims of such subsequent application may be directed to any feature or combination of features described herein. They may take the form of product, composition, process or use claims and may include, by way of example and without limitation, one or more of the following claims:

Claims

1. A polynucleotide sequence which encodes a human papilloma virus (HPV) amino acid sequence, wherein the codon usage pattern of the polynucleotide sequence resembles that of highly expressed mammalian genes.

2. A polynucleotide sequence according to claim 1 in which the codon usage pattern of the polynucleotide sequence is typical of highly expressed human genes.

3. A polynucleotide sequence according to claim 1 or claim 2 in which the codon usage pattern of the polynucleotide sequence also resembles that of highly expressed E.coli genes.

4. A polynucleotide sequence according to any one of claims 1 to 3 which is a DNA sequence.

5. A polynucleotide sequence according to any preceding claim which encodes a HPV polypeptide of HPV type 6, 11, 16, 18, 33 or 45.

6. A polynucleotide sequence according to claim 5 which encodes a HPV polypeptide of HPV type 11, 6a or 6b.

7. A polynucleotide sequence according to any preceding claim which encodes a mutated HPV polypeptide having reduced biological function.

8. A polynucleotide sequence according to claim 7 which encodes a mutated HPV polypeptide comprising one or more point mutations by which one or more of the polypeptide's natural biological functions is inactivated.

9. A polynucleotide sequence according to any preceding claim in which the encoded HPV polypeptide is the whole or a part of a HPV early gene product.

10. A polynucleotide sequence according to claim 9 in which the encoded HPV polypeptide is the whole or a part of E1 or E2.

11. A polynucleotide sequence according to any preceding claim having a codon usage coefficient for highly expressed human genes of greater than 0.3.

12. A polynucleotide sequence according to claim 11 having a codon usage coefficient for highly expressed human genes of greater than 0.4.

13. A polynucleotide sequence according to claim 12 having a codon usage coefficient for highly expressed human genes of greater than 0.5.

14. A polynucleotide sequence according to any preceding claim having a codon usage coefficient for highly expressed E.coli genes of greater than 0.6.

15. A polynucleotide sequence as set out in Fig. 5, or a fragment or variant thereof which maintains the codon usage pattern thereof.

16. A polynucleotide sequence as set out in Fig. 6, or a fragment or variant thereof which maintains the codon usage pattern thereof.

17. An expression vector comprising a polynucleotide sequence according to any preceding claim operably linked to a control sequence which is capable of providing for the expression of the polynucleotide sequence by a host cell.

18. An expression vector according to claim 17 which is capable of directing the expression of the polynucleotide sequence in bacterial insect or mammalian cells.

19. An expression vector according to claim 17 or claim 18 which is p7313PLc.

20. A host cell comprising a polynucleotide sequence according to any one of claims 1-16.

21. A host cell comprising a vector according to any one of claims 17-19.

22. A host cell according to claim 20 or claim 21 which is a bacterial, mammalian, or insect cell.

23. A vaccine composition comprising a polynucleotide sequence according to any one of claims 1-16.

24. A vaccine composition comprising a vector according to any one of claims 17-19.

25. A vaccine composition according to claim 23 or claim 24 comprising a plurality of particles, preferably gold particles, coated with DNA.

26. A vaccine composition according to claim 24 comprising a pharmaceutically acceptable excipient and the DNA vector.

27. A vaccine composition according to any one of claims 23-26 further comprising an adjuvant.

28. The use of a polynucleotide according to any one of claims 1-16 in the treatment or prophylaxis of an HPV infection.

29. The use of a vector according to any one of claims 17-19 in the treatment or prophylaxis of an HPV infection.

30. The use of a vaccine composition according to any one of claims 23-27 in the treatment or prophylaxis of an HPV infection.

31. The use according to any one of claims 28-30 in which the HPV infection is an infection of HPV type 6, 11, 16 or 18.

32. The use of a polynucleotide according to any one of claims 1-16, a vector according to any one of claims 17-19 or a vaccine composition according to any one of claims 23-27 in the treatment or prophylaxis of cutaneous (skin) warts, genital warts, atypical squamous cells of undetermined significance (ASCUS), cervical dysplasia, cervical intraepithelial neoplasia (CIN) or cervical cancer.

33. A method of treating or preventing HPV infections or any symptoms or diseases associated therewith, comprising administering an effective amount of a polynucleotide according to any one of claims 1-16, a vector according to any one of claims 17-19 or a vaccine according to any one of claims 23-27.

34. A method of treating or preventing HPV infections or any symptoms or diseases associated therewith, comprising administering a vaccine according to any one of claims 23-27 in a prime-boost dosage regime with a recombinant viral vector comprising a polynucleotide according to any one of claims 1-16.

Papilloma Virus Sequences

Abstract

The present invention relates to methods and compositions useful in the treatment and prevention of human papilloma virus infections and the symptoms and diseases associated therewith. More particularly, the invention relates to polynucleotide sequences which encode human papilloma virus (HPV) amino acid amino acid sequences, wherein the codon usage pattern of the polynucleotide sequences resemble those of highly expressed mammalian genes.

Fig. 1

```
      1                                                          60
Hpv11-e1    MADDSGTENEGSGCTGWFMVEAIVEHTTGTQISEDEEEEVEDSGYDMVDFIDDRHITQNS
Hpv6a-e1    MADDSGTENEGSGCTGWFMVEAIVQHPTGTQISDDEDEEVEDSGYDMVDFIDDSNITHNS
   6b-e1    MADDSGTENEGSGCTGWFMVEAIVQHPTGTQISDDEDEEVEDSGYDMVDFIDDSNITHNS

             61                                                        120
Hpv11-e1    VEAQALFNRQEADAHYATVQDLKRKYLGSPYVSPISNVANAVESEISPRLDAIKLTTQPK
Hpv6a-e1    LEAQALFNRQEADTHYATVQDLKRKYLGSPYVSPINTIAEAVESEISPRLDAIKLTRQPK
   6b-e1    LEAQALFNRQEADTHYATVQDLKRKYLGSPYVSPINTIAEAVESEISPRLDAIKLTRQPK

             121                                                       180
Hpv11-e1    KVKRRLFETRELTDSGYGYSEVEA..ATQVEKHGDPENGGDGQERDTGRDIEGEGVEHRE
Hpv6a-e1    KVKRRLFQTRELTDSGYGYSEVEAGTGTQVEKHGVPENGGDGQEKDTGRDIEG..EEHTE
   6b-e1    KVKRRLFQTRELTDSGYGYSEVEAGTGTQVEKHGVPENGGDGQEKDTGRDIEG..EEHTE

             181                                                       240
Hpv11-e1    AEAVDDSTREHADTSGILELLKCKDIRSTLHGKFKDCFGLSFVDLIRPFKSDRTTCADWV
Hpv6a-e1    AEAPTNSVREHAGTAGILELLKCKDLRAALLGKFKECFGLSFIDLIRPFKSDKTTCADWV
   6b-e1    AEAPTNSVREHAGTAGILELLKCKDLRAALLGKFKECFGLSFIDLIRPFKSDKTTCLDWV

             241                                                       300
Hpv11-e1    VAGFGIHHSIADAFQKLIEPLSLYAHIQWLTNAWGMVLLVLIRFKVNKSRCTVARTLGTL
Hpv6a-e1    VAGFGIHHSISEAFQKLIEPLSLYAHIQWLTNAWGMVLLVLVRFKVNKSRSTVARTLATL
   6b-e1    VAGFGIHHSISEAFQKLIEPLSLYAHIQWLTNAWGMVLLVLLRFKVNKSRSTVARTLATL

             301                                                       360
Hpv11-e1    LNIPENHMLIEPPKIQSGVRALYWFRTGISNASTVIGEAPEWITRQTVIEHSLADSQFKL
Hpv6a-e1    LNIPDNQMLIEPPKIQSGVAALYWFRTGISNASTVIGEAPEWITRQTVIEHGLADSQFKL
   6b-e1    LNIPENQMLIEPPKIQSGVAALYWFRTGISNASTVIGEAPEWITRQTVIEHGLADSQFKL

             361                                                       420
Hpv11-e1    TEMVQWAYDNDICEESEIAFEYAQRGDFDSNARAFLNSNMQAKYVKDCAIMCRHYKHAEM
Hpv6a-e1    TEMVQWAYDNDICEESEIAFEYAQRGDFDSNARAFLNSNMQAKYVKDCATMCRHYKHAEM
   6b-e1    TEMVQWAYDNDICEESEIAFEYAQRGDFDSNARAFLNSNMQAKYVKDCATMCRHYKHAEM

             421                                                       480
Hpv11-e1    KKMSIKQWIKYRGTKVDSVGNWKPIVQFLRHQNIEFIPFLSKLKLWLHGTPKKNCIAIVG
Hpv6a-e1    RKMSIKQWIKHRGSKIEGTGNWKPIVQFLRHQNIEFIPFLSKFKLWLHGTPKKNCIAIVG
   6b-e1    RKMSIKQWIKHRGSKIEGTGNWKPIVQFLRHQNIEFIPFLTKFKLWLHGTPKKNCIAIVG

             481                                                       540
Hpv11-e1    PPDTGKSCFCMSLIKFLGGTVISYVNSCSHFWLQPLTDAKVALLDDATQPCWTYMDTYMR
Hpv6a-e1    PPDTGKSYFCMSLISFLGGTVISHVNSSSHFWLQPLVDAKVALLDDATQPCWIYMDTYMR
   6b-e1    PPDTGKSYFCMSLISFLGGTVISHVNSSSHFWLQPLVDAKVALLDDATQPCWIYMDTYMR

             541                                                       600
Hpv11-e1    NLLDGNPMSIDRKHRALTLIKCPPLLVTSNIDISKEEKYKYLHSRVTTFTFPNPFPFDRN
Hpv6a-e1    NLLDGNPMSIDRKHKALTLIKCPPLLVTSNIDITKEEKYKYLHTRVTTFTFPNPFPFDRN
   6b-e1    NLLDGNPMSIDRKHKALTLIKCPPLLVTSNIDITKEDKYKYLHTRVTTFTFPNPFPFDRN

             601                                                       657
Hpv11-e1    GNAVYELSDANWKCFFERLSSSLDIEDSEDEEDGSNSQAFRCVPGSVVRTL
Hpv6a-e1    GNAVYELSNANWKCFFERLSSSLDIQDSEDEEDGSNSQAFRCVPGTVVRTL
   6b-e1    GNAVYELSNTNWKCFFERLSSSLDIQDSEDEEDGSNSQAFRCVPGTVVRTL
```

Fig. 2

```
            1                                                          60
6b-e1       MADDSGTENEGSGCTGWFMVEAIVQHPTGTQISDDEDEEVEDSGYDMVDFIDDSNITHNS
6b-e1 mut   MADDSGTENEGSGCTGWFMVEAIVQHPTGTQISDDEDEEVEDSGYDMVDFIDDSNITHNS

            61                                                         120
6b-e1       LEAQALFNRQEADTHYATVQDLKRKYLGSPYVSPINTIAEAVESEISPRLDAIKLTRQPK
6b-e1 mut   LEAQALFNRQEADTHYATVQDLGGKYLGSPYVSPINTIAEAVESEISPRLDAIKLTRQPK

            121                                                        180
6b-e1       KVKRRLFQTRELTDSGYGYSEVEAGTGTQVEKHGVPENGGDGQEKDTGRDIEG..EEHTE
6b-e1 mut   KVKRRLFQTRELTDSGYGYSEVEAGTGTQVEKHGVPENGGDGQEKDTGRDIEG..EEHTE

            181                                                        240
6b-e1       AEAPTNSVREHAGTAGILELLKCKDLRAALLGKFKECFGLSFIDLIRPFKSDKTTCLDWV
6b-e1 mut   AEAPTNSVREHAGTAGILELLKCKDLRAALLGKFKECFGLSFIDLIRPFKSDKTTCLDWV

            241                                                        300
6b-e1       VAGFGIHHSISEAFQKLIEPLSLYAHIQWLTNAWGMVLLVLLRFKVNKSRSTVARTLATL
6b-e1 mut   VAGFGIHHSISEAFQKLIEPLSLYAHIQWLTNAWGMVLLVLLRFKVNKSRSTVARTLATL

            301                                                        360
6b-e1       LNIPENQMLIEPPKIQSGVAALYWFRTGISNASTVIGEAPEWITRQTVIEHGLADSQFKL
6b-e1 mut   LNIPENQMLIEPPKIQSGVAALYWFRTGISNASTVIGEAPEWITRQTVIEHGLADSQFKL

            361                                                        420
6b-e1       TEMVQWAYDNDICEESEIAFEYAQRGDFDSNARAFLNSNMQAKYVKDCATMCRHYKHAEM
6b-e1 mut   TEMVQWAYDNDICEESEIAFEYAQRGDFDSNARAFLNSNMQAKYVKDCATMCRHYKHAEM

            421                                                        480
6b-e1       RKMSIKQWIKHRGSKIEGTGNWKPIVQFLRHQNIEFIPFLTKFKLWLHGTPKKNCIAIVG
6b-e1 mut   RKMSIKQWIKHRGSKIEGTGNWKPIVQFLRHQNIEFIPFLTKFKLWLHGTPKKNCIAIVG

            481                                                        540
6b-e1       PPDTGKSYFCMSLISFLGGTVISHVNSSSHFWLQPLVDAKVALLDDATQPCWIYMDTYMR
6b-e1 mut   PPDTGKSYFCMSLISFLGGTVISHVNSSSHFWLQPLVDAKVALLDDATQPCWIYMDTYMR

            541                                                        600
6b-e1       NLLDGNPMSIDRKHKALTLIKCPPLLVTSNIDITKEDKYKYLHTRVTTFTFPNPFPFDRN
6b-e1 mut   NLLDGNPMSIDRKHKALTLIKCPPLLVTSNIDITKEDKYKYLHTRVTTFTFPNPFPFDRN

            601                                                        657
6b-e1       GNAVYELSNTNWKCFFERLSSSLDIQDSEDEEDGSNSQAFRCVPGTVVRTL
6b-e1 mut   GNAVYELSNTNWKCFFERLSSSLDIQDSEDEEDGSNSQAFRCVPGTVVRTL
```

Fig. 3

```
            1                                                          60
Hpv-11e2    MEAIAKRLDACQDQLLELYEENSIDIHKHIMHWKCIRLESVLLHKAKQMGLSHIGLQVVP
Hpv6a-e2    MEAIAKRLDACQEQLLELYEENSTDLNKHVLHWKCMRHESVLLYKAKQMGLSHIGMQVVP
Hpv6b-e2    MEAIAKRLDACQEQLLELYEENSTDLHKHVLHWKCMRHESVLLYKAKQMGLSHIGMQVVP

            61                                                         120
Hpv-11e2    PLTVSETKGHNAIEMQMHLESLAKTQYGVEPWTLQDTSYEMWLTPPKRCFKKQGNTVEVK
Hpv6a-e2    PLKVSEAKGHNAIEMQMHLESLLKTEYSMEPWTLQETSYEMWQTPPKRCFKKRGKTVEVK
Hpv6b-e2    PLKVSEAKGHNAIEMQMHLESLLRTEYSMEPWTLQETSYEMWQTPPKRCFKKRGKTVEVK

            121                                                        180
Hpv-11e2    FDGCEDNVMEYVVWTHIYLQDNDSWVKVTSSVDAKGIYYTCGQFKTYYVNFNKEAQKYGS
Hpv6a-e2    FDGCANNTMDYVVWTDVYVQDTDSWVKVHSMVDAKGIYYTCGQFKTYYVNFVKEAEKYGS
Hpv6b-e2    FDGCANNTMDYVVWTDVYVQDNDTWVKVHSMVDAKGIYYTCGQFKTYYVNFVKEAEKYGS

            181                                                        240
Hpv-11e2    TNHWEVCYGSTVICSPASVSSTVREVSIAEPTTYTPAQTTAPTVSACTTEDGVSAPPRKR
Hpv6a-e2    TKQWEVCYGSTVICSPASVSSTTQEVSIPESTTYTPAQTSTP.VSSSTQEDAVQTPPRKR
Hpv6b-e2    TKHWEVCYGSTVICSPASVSSTTQEVSIPESTTYTPAQTSTL.VSSSTKEDAVQTPPRKR

            241                                                        300
Hpv-11e2    ARGPSTN..NTLCVANIRSVDSTINNIVTDNYNKHQRRNNCHSAATPIVQLQGDSNCLKC
Hpv6a-e2    ARGVQQSPCNALCVAHIGPVDSGNHNLITNNHDQHQRRNNSNSSATPIVQFQGESNCLKC
Hpv6b-e2    ARGVQQSPCNALCVAHIGPVDSGNHNLITNNHDQHQRRNNSNSSATPIVQFQGESNCLKC

            301                                                        360
Hpv-11e2    FRYRLNDKYKHLFELASSTWHWASPEAPHKNAIVTLTYSSEEQRQQFLNSVKIPPTIRHK
Hpv6a-e2    FRYRLNDKHRHLFDLISSTWHWASPKAPHKHAIVTVTYHSEEQRQQFLNVVKIPPTIRHK
Hpv6b-e2    FRYRLNDRHRHLFDLISSTWHWASSKAPHKHAIVTVTYDSEEQRQQFLDVVKIPPTISHK

            361             373
Hpv-11e2    VGFMSLHLL
Hpv6a-e2    LGFMSLHLL
Hpv6b-e2    LGFMSLHLL
```

Fig. 4

```
            1                                                          60
Hpv-11e2    MEAIAKRLDACQDQLLELYEENSIDIHKHIMHWKCIRLESVLLHKAKQMGLSHIGLQVVP
Hpv-11e2mut MEAIAKRLDACQDQLLELYEENSIDIHKHIMHWKCIRLESVLLHKAKQMGLSHIGLQVVP

            61                                                         120
Hpv-11e2    PLTVSETKGHNAIEMQMHLESLAKTQYGVEPWTLQDTSYEMWLTPPKRCFKKQGNTVEVK
Hpv-11e2mut PLTVSETKGHNAIEMQMHLESLAKTQYGVEPWTLQDTSYEMWLTPPKRCFAKQGNTVEVK

            121                                                        180
Hpv-11e2    FDGCEDNVMEYVVWTHIYLQDNDSWVKVTSSVDAKGIYYTCGQFKTYYVNFNKEAQKYGS
Hpv-11e2mut FDGCEDNVMEYVVWTHIYLQDNDSWVKVTSSVDAKGIYYTCGQFKTYYVNFNKEAQKYGS

            181                                                        240
Hpv-11e2    TNHWEVCYGSTVICSPASVSSTVREVSIAEPTTYTPAQTTAPTVSACTTEDGVSAPPRKR
Hpv-11e2mut TNHWEVCYGSTVICSPASVSSTVREVSIAEPTTYTPAQTTAPTVSACTTEDGVSAPPRKR

            241                                                        300
Hpv-11e2    ARGPSTN..NTLCVANIRSVDSTINNIVTDNYNKHQRRNNCHSAATPIVQLQGDSNCLKC
Hpv-11e2mut ARGPSTN..NTLCVANIRSVDSTINNIVTDNYNKHQRRNNCHSAATPIVQLQGDSNCLKC

            301                                                        360
Hpv-11e2    FRYRLNDKYKHLFELASSTWHWASPEAPHKNAIVTLTYSSEEQRQQFLNSVKIPPTIRHK
Hpv-11e2mut FRYRLNDKYKHLFELASSTWHWASPEAPHKNAIVTLTYSSEEQRQQFLNSVKIPPTIRHK

            361              373
Hpv-11e2    VGFMSLHLL
Hpv-11e2mut VGFMSLHLL


            1                                                          60
Hpv6b-e2    MEAIAKRLDACQEQLLELYEENSTDLHKHVLHWKCMRHESVLLYKAKQMGLSHIGMQVVP
Hpv6b-e2    MEAIAKRLDACQEQLLELYEENSTDLHKHVLHWKCMRHESVLLYKAKQMGLSHIGMQVVP

            61                                                         120
Hpv6b-e2    PLKVSEAKGHNAIEMQMHLESLLRTEYSMEPWTLQETSYEMWQTPPKRCFKKRGKTVEVK
Hpv6b-e2    PLKVSEAKGHNAIEMQMHLESLLRTEYSMEPWTLQETSYEMWQTPPKRCFKKRGKTVEVK

            121                                                        180
Hpv6b-e2    FDGCANNTMDYVVWTDVYVQDNDTWVKVHSMVDAKGIYYTCGQFKTYYVNFVKEAEKYGS
Hpv6b-e2    FDGCANNTMDYVVWTDVYVQDNDTWVKVHSMVDAKGIYYTCGQFKTYYVNFVKEAEKYGS

            181                                                        240
Hpv6b-e2    TKHWEVCYGSTVICSPASVSSTTQEVSIPESTTYTPAQTSTL.VSSSTKEDAVQTPPRKR
Hpv6b-e2    TKHWEVCYGSTVICSPASVSSTTQEVSIPESTTYTPAQTSTL.VSSSTKEDAVQTPPRKR

            241                                                        300
Hpv6b-e2    ARGVQQSPCNALCVAHIGPVDSGNHNLITNNHDQHQRRNNSNSSATPIVQFQGESNCLKC
Hpv6b-e2    ARGVQQSPCNALCVAHIGPVDSGNHNLITNNHDQHQRRNNSNSSATPIVQFQGESNCLKC

            301                                                        360
Hpv6b-e2    FRYRLNDRHRHLFDLISSTWHWASSKAPHKHAIVTVTYDSEEQRQQFLDVVKIPPTISHK
Hpv6b-e2    FRYRLNDRHRHLFDLISSTWHWASSKAPHKHAIVTVTYDSEEQRQQFLDVVKIPPTISHK

            361              373
Hpv6b-e2    LGFMSLHLL
Hpv6b-e2    LGFMSLHLL
```

Fig. 5

```
          1                                                          60
w-t el    GGCCGCCATGGCGGACGATTCAGGTACAGAAAATGAGGGGTCTGGGTGTACAGGATGGTT
Syn el    GGCCGCCATGGCAGACGATTCCGGTACTGAGAACGAAGGTTCTGGTTGTACCGGTTGGTT

          61                                                        120
w-t el    TATGGTAGAAGCTATAGTGCAACACCCAACAGGTACACAAATATCAGACGATGAGGATGA
Syn el    CATGGTTGAAGCAATCGTTCAGCATCCGACTGGTACCCAGATCTCCGATGACGAAGACGA

          121                                                       180
w-t el    GGAGGTGGAGGACAGTGGGTATGACATGGTGGACTTTATTGATGACAGCAATATTACACA
Syn el    AGAAGTTGAAGATTCTGGTTACGACATGGTTGACTTCATCGATGACTCCAACATCACTCA

          181                                                       240
w-t el    CAATTCACTGGAAGCACAGGCATTGTTTAACAGGCAGGAGGCGGACACCCATTATGCGAC
Syn el    TAACTCTCTGGAAGCACAGGCTCTGTTTAACCGCCAGGAAGCTGATACCCATTACGCTAC

          241                                                       300
w-t el    TGTGCAGGACCTAAAACGAAAGTATTTAGGTAGTCCATATGTTAGTCCTATAAACACTAT
Syn el    TGTTCAGGACCTGAAACGCAAATATCTGGGCTCTCCGTACGTTTCCCCGATCAACACTAT

          301                                                       360
w-t el    AGCCGAGGCAGTGGAAAGTGAAATAAGTCCACGATTGGACGCCATTAAACTTACAAGACA
Syn el    CGCAGAAGCAGTTGAGTCTGAAATCTCCCCGCGCCTGGACGCTATCAAACTGACTCGTCA

          361                                                       420
w-t el    GCCAAAAAAGGTAAAGCGACGGCTGTTTCAAACCAGGGAACTAACGGACAGTGGATATGG
Syn el    GCCGAAGAAGGTTAAACGTCGTCTGTTCCAGACTCGTGAACTGACCGACTCCGGTTACGG

          421                                                       480
w-t el    CTATTCTGAAGTGGAAGCTGGAACGGGAACGCAGGTAGAGAAACATGGCGTACCGGAAAA
Syn el    TTATAGCGAAGTTGAGGCTGGCACCGGCACCCAGGTTGAAAAACACGGTGTACCGGAAAA

          481                                                       540
w-t el    TGGGGGAGATGGTCAGGAAAAGGACACAGGAAGGGACATAGAGGGGGAGGAACATACAGA
Syn el    CGGCGGCGACGGTCAGGAAAAGGACACCGGCCGCGACATCGAGGGTGAGGAACACACCGA

          541                                                       600
w-t el    GGCGGAAGCGCCCACAAACAGTGTACGGGAGCATGCAGGCACAGCAGGAATATTGGAATT
Syn el    AGCTGAAGCTCCGACTAACTCTGTTCGTGAACACGCAGGTACTGCGGGTATCCTGGAACT

          601                                                       660
w-t el    GTTAAAATGTAAAGATTTACGGGCAGCATTACTTGGTAAGTTTAAAGAATGCTTTGGGCT
Syn el    GCTGAAATGCAAAGACCTGCGCGCGGCTCTGCTGGGCAAATTCAAAGAATGCTTCGGCCT

          661                                                       720
w-t el    GTCTTTTATAGATTTAATTAGGCCATTTAAAAGTGATAAAACAACATGTTTAGATTGGGT
Syn el    GTCTTTCATTGACCTGATCCGTCCGTTTAAGTCTGACAAAACTACCTGTCTGGACTGGGT

          721                                                       780
w-t el    GGTAGCAGGGTTTGGTATACATCATAGCATATCAGAGGCATTTCAAAAATTAATTGAGCC
Syn el    TGTAGCAGGCTTCGGCATCCACCACTCTATCTCTGAAGCATTCCAGAAACTGATCGAGCC

          781                                                       840
w-t el    ATTAAGTTTATATGCACATATACAATGGCTAACAAATGCATGGGGAATGGTATTGTTAGT
```

Fig. 5 Cont.

```
Syn e1  GCTGTCTCTGTACGCGCACATCCAGTGGCTGACTAACGCTTGGGGTATGGTTCTGCTGGT

        841                                                       900
w-t e1  ATTATTAAGATTTAAAGTAAATAAAAGTAGAAGTACCGTTGCACGTACACTTGCAACGCT
Syn e1  ACTGCTGCGCTTTAAAGTAAACAAATCTCGTTCCACTGTTGCTCGTACTCTGGCTACCCT

        901                                                       960
w-t e1  ATTAAATATACCTGAAAACCAAATGTTAATAGAGCCACCAAAAATACAAAGTGGTGTTGC
Syn e1  GCTGAACATCCCGGAGAACCAGATGCTGATCGAACCGCCGAAAATCCAGTCTGGTGTAGC

        961                                                      1020
w-t e1  AGCCCTGTATTGGTTTCGTACAGGTATATCAAATGCCAGTACAGTTATAGGGGAAGCACC
Syn e1  TGCACTGTACTGGTTTCGTACTGGCATCTCTAACGCTAGCACTGTTATCGGTGAAGCACC

        1021                                                     1080
w-t e1  AGAATGGATAACACGCCAAACAGTTATTGAACACGGGTTGGCAGACAGTCAGTTTAAATT
Syn e1  GGAATGGATCACTCGTCAGACCGTTATCGAACACGGTCTGGCAGATTCTCAGTTCAAACT

        1081                                                     1140
w-t e1  AACAGAAATGGTGCAGTGGGCGTATGATAATGACATATGCGAGGAGAGTGAAATTGCATT
Syn e1  GACTGAAATGGTTCAGTGGGCATACGACAACGACATCTGCGAGGAATCTGAAATTGCGTT

        1141                                                     1200
w-t e1  TGAATATGCACAAAGGGGAGATTTTGATTCTAATGCACGAGCATTTTTAAATAGCAATAT
Syn e1  CGAATACGCTCAGCGTGGCGACTTCGACTCCAACGCTCGTGCTTTCCTGAACAGCAACAT

        1201                                                     1260
w-t e1  GCAGGCAAAATATGTGAAAGATTGTGCAACTATGTGTAGACATTATAAACATGCAGAAAT
Syn e1  GCAGGCTAAATACGTAAAAGACTGCGCTACCATGTGCCGTCACTACAAACACGCGGAAAT

        1261                                                     1320
w-t e1  GAGGAAGATGTCTATAAAACAATGGATAAAACATAGGGGTTCTAAAATAGAAGGCACAGG
Syn e1  GCGTAAAATGTCTATCAAACAGTGGATCAAGCACCGCGGTTCTAAAATCGAAGGTACCGG

        1321                                                     1380
w-t e1  AAATTGGAAACCAATTGTACAATTCCTACGACATCAAAATATAGAATTCATTCCTTTTTT
Syn e1  TAACTGGAAACCGATCGTTCAGTTCCTGCGCCATCAGAACATCGAATTCATCCCGTTCCT

        1381                                                     1440
w-t e1  AACTAAATTTAAATTATGGCTGCACGGTACGCCAAAAAAAAACTGCATAGCCATAGTAGG
Syn e1  GACCAAATTCAAGCTGTGGCTGCACGGTACCCCGAAAAAAAACTGCATCGCTATCGTAGG

        1441                                                     1500
w-t e1  CCCTCCAGATACTGGGAAATCGTACTTTTGTATGAGTTTAATAAGCTTTCTAGGAGGTAC
Syn e1  TCCACCGGACACTGGCAAGTCTTACTTCTGTATGTCCCTGATCTCTTTCCTGGGCGGCAC

        1501                                                     1560
w-t e1  AGTTATTAGTCATGTAAATTCCAGCAGCCATTTTTGGTTGCAACCGTTAGTAGATGCTAA
Syn e1  TGTAATCTCTCACGTTAACTCTTCCTCCCATTTCTGGCTGCAGCCACTGGTAGACGCGAA

        1561                                                     1620
w-t e1  GGTAGCATTGTTAGATGATGCAACACAGCCATGTTGGATATATATGGATACATATATGAG
Syn e1  AGTAGCTCTGCTGGACGACGCGACCCAGCCGTGCTGGATCTACATGGATACTTACATGCG

        1621                                                     1680
w-t e1  AAATTTGTTAGATGGTAATCCTATGAGTATTGACAGAAAGCATAAAGCATTGACATTAAT
Syn e1  CAACCTGCTGGACGGTAACCCGATGTCTATCGACCGTAAACACAAAGCGCTGACTCTGAT
```

Fig. 5 Cont.

```
        1681                                                        1740
w-t e1  TAAATGTCCACCTCTGCTAGTAACGTCCAACATAGATATTACTAAAGAAGATAAATATAA
Syn e1  CAAGTGCCCGCCGCTGCTGGTAACTTCTAACATCGACATCACCAAGGAAGATAAATACAA

        1741                                                        1800
w-t e1  GTATTTACATACTAGAGTAACAACATTTACATTTCCAAATCCATTCCCTTTTGACAGAAA
Syn e1  GTACCTGCATACCCGTGTTACTACCTTTACTTTCCCGAACCCGTTCCCGTTTGATCGTAA

        1801                                                        1860
w-t e1  TGGGAATGCAGTGTATGAACTGTCAAATACAAACTGGAAATGTTTTTTTGAAAGACTGTC
Syn e1  CGGTAACGCTGTTTACGAACTGTCCAACACTAACTGGAAATGCTTCTTCGAGCGTCTGTC

        1861                                                        1920
w-t e1  GTCAAGCCTAGACATTCAGGATTCTGAGGACGAGGAAGATGGAAGCAATAGCCAAGCGTT
Syn e1  TTCCTCCCTGGACATCCAGGACTCTGAAGATGAAGAAGATGGTTCTAACTCTCAGGCTTT

        1921                                                        1980
w-t e1  TAGATGCGTGCCAGGAACAGTTGTTAGAACTTTAGACCATCATCACCATCACCATTGATG
Syn e1  CCGTTGTGTTCCGGGTACTGTTGTTCGTACTCTGTGA.....................G

        1981       1984
w-t e1  ''''
Syn e1  ''''
```

Fig. 6

```
                1                                                          60
Hpv11 e2 mut    ''GCGGCCGCCATGGAAGCCATCGCGAAGAGGCTCGACGCCTGCCAGGACCAGCTGCTCG

               61                                                         120
Hpv11 e2 mut    AGCTGTACGAGGAGAACAGCATTGACATCCATAAGCACATCATGCACTGGAAGTGCATTC

              121                                                         180
Hpv11 e2 mut    GCCTGGAGAGCGTGCTGTTGCACAAGGCCAAGCAGATGGGCCTGTCCCACATAGGCCTTC

              181                                                         240
Hpv11 e2 mut    AGGTGGTCCCCCCTCTGACCGTGTCAGAGACAAAGGGCCATAACGCAATCGAGATGCAGA

              241                                                         300
Hpv11 e2 mut    TGCACCTCGAGTCGCTGGCGAAAACACAGTACGGCGTGGAGCCATGGACCCTGCAGGACA

              301                                                         360
Hpv11 e2 mut    CCTCGTACGAAATGTGGCTGACCCCACCTAAGCGATGCTTCGCCAAACAGGGCAACACAG

              361                                                         420
Hpv11 e2 mut    TGGAGGTGAAGTTCGACGGCTGTGAGGATAACGTTATGGAGTATGTCGTGTGGACGCACA

              421                                                         480
Hpv11 e2 mut    TCTATCTGCAGGACAACGACAGTTGGGTGAAGGTGACCAGCTCCGTGGACGCGAAGGGCA

              481                                                         540
Hpv11 e2 mut    TCTACTATACCTGTGGGCAGTTTAAAACCTACTATGTGAACTTCAACAAAGAGGCCCAAA

              541                                                         600
Hpv11 e2 mut    AGTATGGCTCCACCAACCACTGGGAGGTCTGCTATGGGAGCACGGTGATTTGCTCTCCCG

              601                                                         660
Hpv11 e2 mut    CCAGCGTGTCTAGCACTGTGCGCGAGGTGAGCATTGCCGAGCCGACCACGTACACCCCTG

              661                                                         720
Hpv11 e2 mut    CCCAGACGACCGCTCCGACCGTGTCTGCTTGTACTACCGAGGACGGCGTGAGCGCTCCAC

              721                                                         780
Hpv11 e2 mut    CCAGGAAGCGTGCGAGGGGCCCAAGCACCAACAACACCCTCTGTGTGGCGAACATTCGCA

              781                                                         840
Hpv11 e2 mut    GCGTCGACAGTACCATCAATAACATCGTGACGGATAACTATAACAAGCACCAGAGGCGTA

              841                                                         900
Hpv11 e2 mut    ACAACTGTCACTCTGCCGCAACCCCCATCGTGCAGCTCCAGGGAGACAGCAATTGCCTTA

              901                                                         960
Hpv11 e2 mut    AGTGCTTCCGCTATCGCCTCAACGACAAGTACAAGCACCTCTTTGAGCTCGCCTCGTCGA

              961                                                        1020
Hpv11 e2 mut    CGTGGCACTGGGCCTCACCCGAGGCACCTCACAAGAACGCCATCGTCACTCTCACTTACT

             1021                                                        1080
Hpv11 e2 mut    CCAGTGAGGAGCAGAGACAGCAGTTTCTGAACAGCGTGAAGATCCCACCGACGATCCGTC

             1081                                          1123
Hpv11 e2 mut    ATAAGGTCGGCTTCATGTCACTGCATCTCCTGTGAGGATCC''
```

Figure 7: WRG7313plc

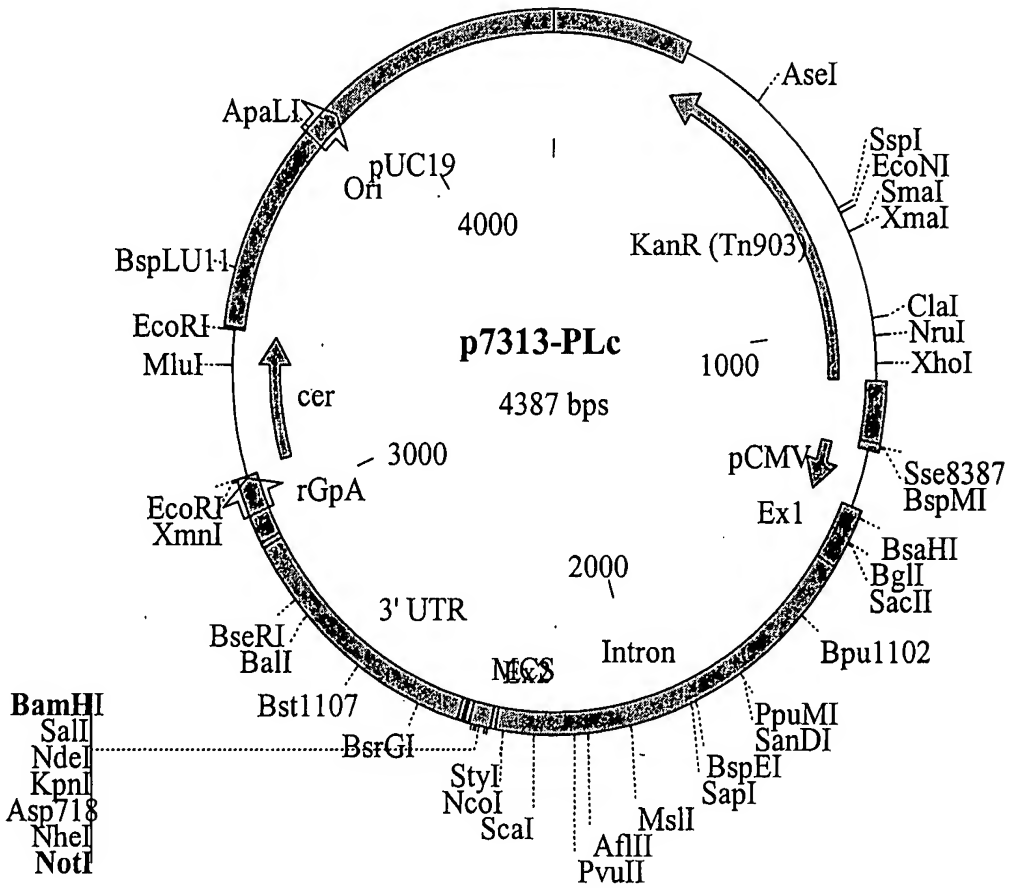
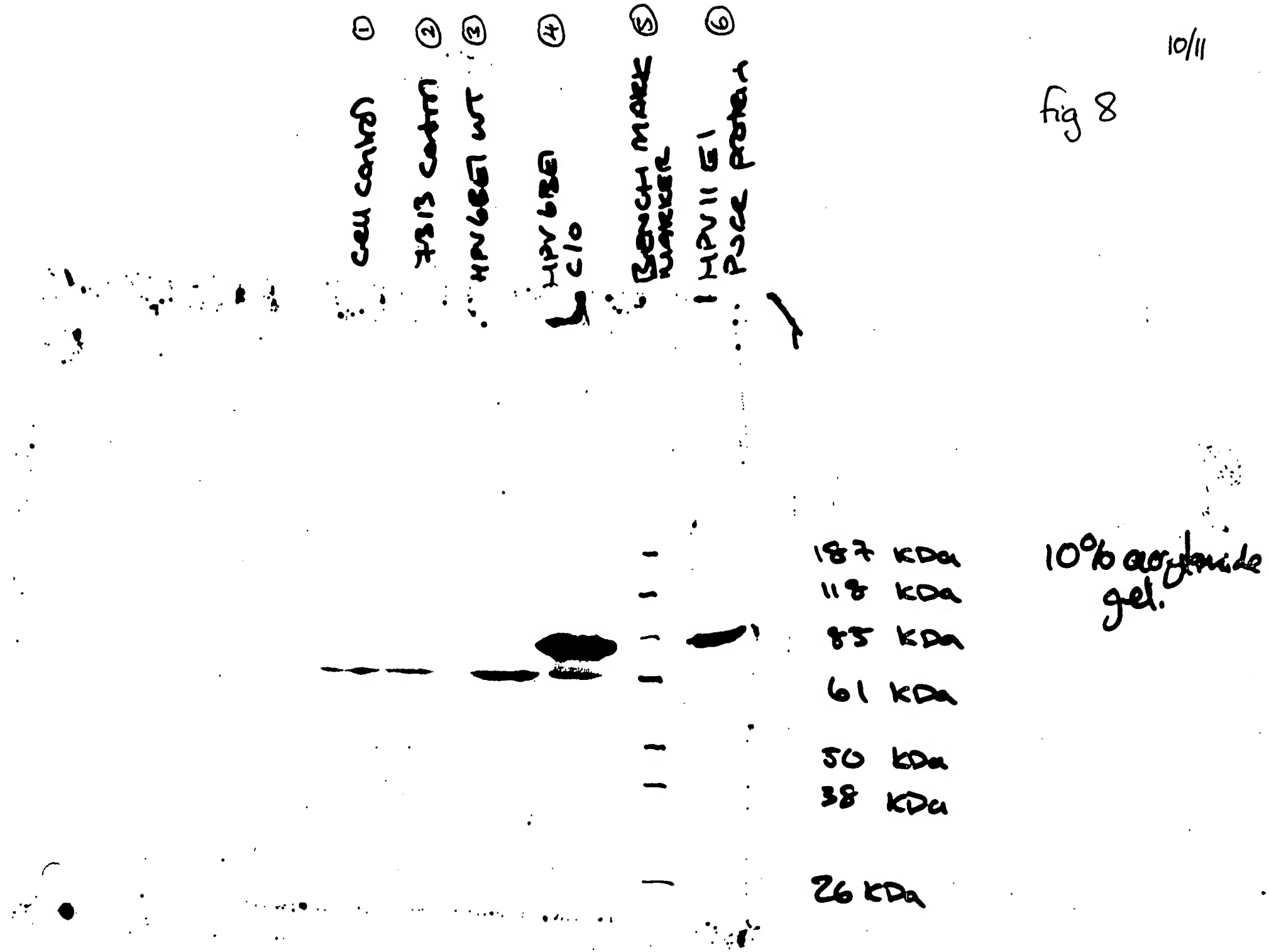


fig 8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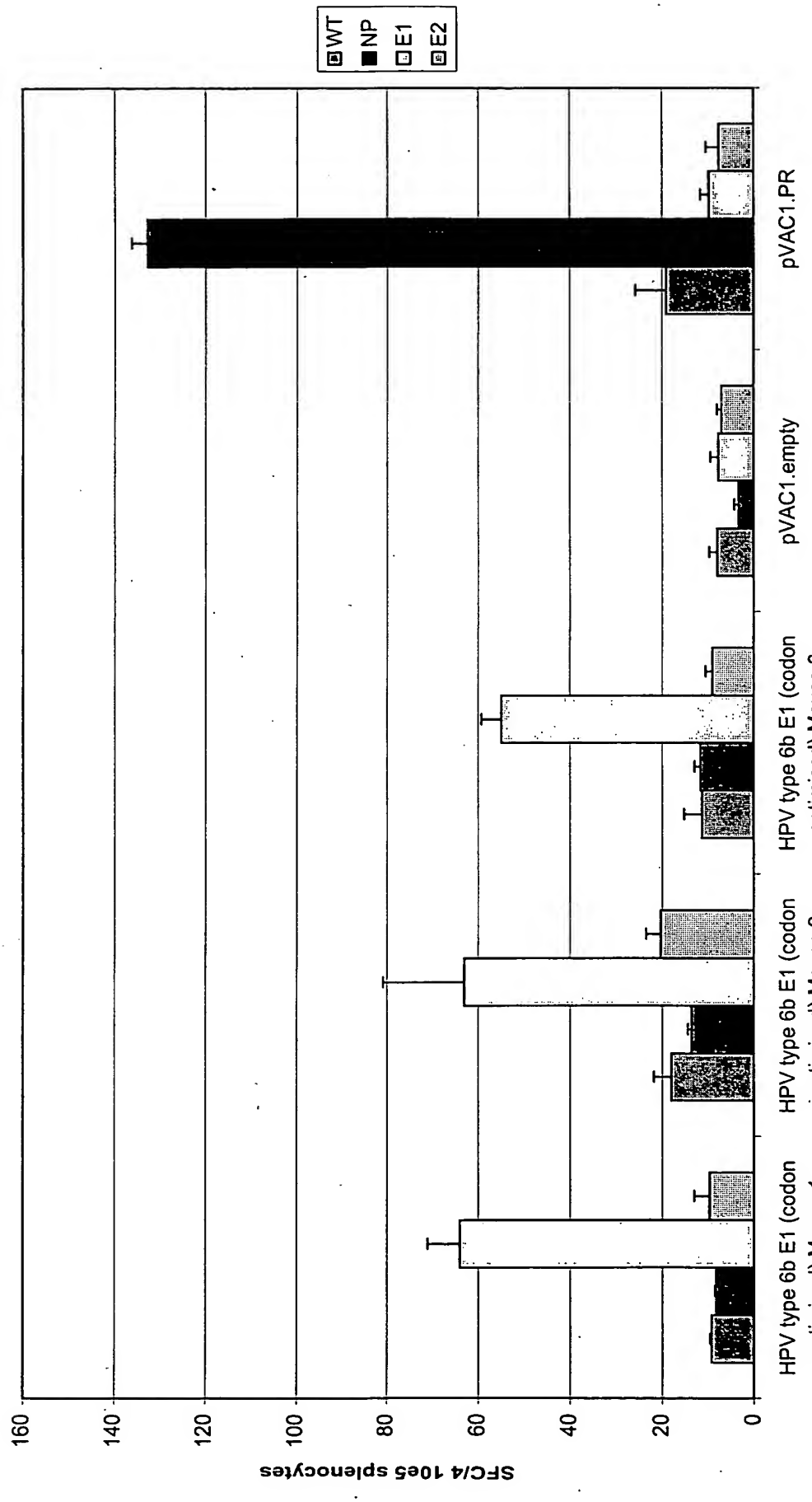
② 293 control
③ HPV16 E6 WT
④ HPV16 E6 C10
⑤ Prestained marker
⑥ HPV11 E6 PSDC protein
187 kDa
118 kDa
85 kDa
61 kDa
50 kDa
38 kDa
26 kDa
10% acrylamide gel.

Fig. 9 KB00/10 IFNg ELISPOT - 2 weeks post boost @ 4 weeks



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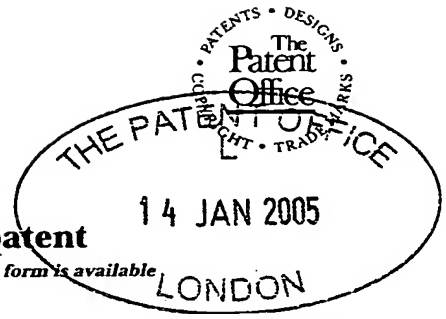

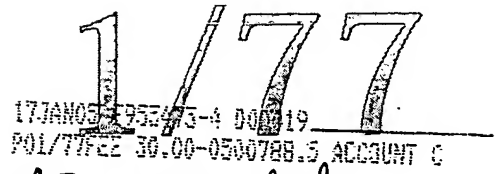

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Application number GB 0500788.5

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2. Full name, address and postcode of the applicant or of each applicant *(underline all surnames)*:

ELEMENT SIX LIMITED
ISLE OF MAN FREEPORT
BALLASALLA
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ISLE OF MAN, U.K.

Patents ADP number *(if you know it)*: ISLE OF MAN, U.K.

If the applicant is a corporate body, give the country/state of its incorporation: 8479958001

3. Title of the invention: RIGID THREE-DIMENSIONAL COMPONENTS

4. Name of your agent *(if you have one)*: CARPMAELS & RANSFORD

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*:
43-45 BLOOMSBURY SQUARE
LONDON
WC1A 2RA

Patents ADP number *(if you know it)*: 83001

5. Priority declaration: Are you claiming priority from one or more earlier-filed patent applications? If so, please give details of the application(s):

Country	Application number *(if you know it)*	Date of filing *(day / month / year)*

6. Divisionals etc: Is this application a divisional application, or being made following resolution of an entitlement dispute about an earlier application? If so, please give the application number and filing date of the earlier application:

Number of earlier UK application	Date of filing *(day / month / year)*

7. Inventorship: (Inventors must be individuals not companies) (Please tick the appropriate boxes)

Are all the applicants named above also inventors? YES ☐ NO ☑

If yes, are there any other inventors? YES ☐ NO ☐

8. Are you paying the application fee with this form? YES ☑ NO ☐

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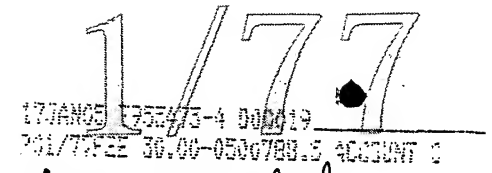

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2. Full name, address and postcode of the applicant or of each applicant *(underline all surnames)*:
ELEMENT SIX LIMITED
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ISLE OF MAN, U.K.

Patents ADP number *(if you know it)*: ISLE OF MAN, U.K.

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Country	Application number *(if you know it)*	Date of filing *(day / month / year)*

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Description: 12

Claim *(s)*: 1

Abstract:

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Request for search (Patents Form 9A/77):

Request for substantive examination (Patents Form 10/77):

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11. I/We request the grant of a patent on the basis of this application.

Signature(s): Carpmaels & Ransford CARPMAELS & RANSFORD Date: 14th January 2005

12. Name, e-mail address, telephone, Fax and/or mobile number, if any, of a contact point for the applicant: Hugh Goodfellow 020 7242 8692

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Rigid Three-Dimensional Components

BACKGROUND TO THE INVENTION

THIS invention relates to rigid three-dimensional components, which have high rigidity and low mass, and in particular to speaker domes.

There are many applications requiring structures of high rigidity and low mass. Typical applications are in the aerospace industry where virtually all mechanical components must have a high rigidity to mass ratio.

However, there is a range of other applications for light but rigid bodies. A particular application is the production of drive units for acoustic loudspeakers, and in particular high frequency tweeters for the accurate reproduction of high frequency sounds.

Human hearing is commonly accepted to cover the range 20 Hz – 20 kHz. Therefore a high quality loudspeaker system needs to accurately reproduce frequencies at least over this frequency range. Typical high performance loudspeakers employ two or more drive units that are effectively mechanical transducers converting an electrical signal into a sound (compression) wave. Each drive unit will cover a specific part of the audible range. The drive unit can be approximated to a piston moving backwards and forwards to create compression and rarefaction of air.

It is well known that small pistons can efficiently generate high sound pressure levels at high frequencies while larger diameter pistons are required to produce comparable sound pressure levels at lower frequencies with comparable efficiency. Typically a 25mm diameter drive unit can operate in the frequency range 2-20kHz while a larger

drive unit of, say, 100-250mm diameter can produce frequencies in the range down to 100Hz and below. However, larger drive units cannot easily be used to produce high frequency sounds due to the problems of unwanted oscillations or break-up that can occur. Human ears are very sensitive to the colouration of the sound by these break-up modes. For this reason high frequency drive units generally have a small diameter. Recently it has been demonstrated that the presence of break-up modes at frequencies that lie outside the accepted range of human hearing can cause audible degradation of the source. For this reason several attempts have been made to produce drive units that can operate at frequencies higher than 20kHz without distortion.

The ideal loudspeaker would have very low mass, to enhance its sensitivity, and very high rigidity with no resonances within or close to the frequency spectrum of operation which could affect the audible output. All practical tweeter devices naturally have mass, and also resonances. Developments in audio media and amplification systems, such as the so called Super Audio formats (SACD and DVDA) extend the range of frequencies provided in the drive to modern speakers up to as high as 96 kHz, compared for example with the upper limit of the bandwidth of a standard CD, which is about 22 kHz.

It is well known that lighter and more rigid tweeter structures, fabricated using materials with a higher value of Young's modulus and lower density, show higher frequency resonances. As such, the use of diamond in tweeters is well reported. Prior art records a variety of configurations of speaker dome, fabricated by a range of means, but the performance advantage reported is generally poor and such speaker domes are not in widespread use. There is also substantial prior art in tweeter devices based on other materials such as Al, Be and plastics, and on a range of geometries.

US Patent 5,556,464 discloses the use of diamond domes for speakers, describing in detail the need to terminate the edge of the integral planar flange in a manner designed to control edge cracks developing. DE Patent 10049744 discloses the use of a diamond dome mounted concave onto a voice coil former, such that the edges of the dome are unsupported. This type of geometry provides for a range of unwanted resonances in the dome structure that may colour the output sound. More recently, Bower and Wilkins (B&W Loudspeakers Ltd, Dale Road, Worthing, West Sussex, England) have launched a

range of speakers using diamond domes, the design of which is described in co-pending GB patent application 0408458.8.

However there are limitations on the use of diamond and other stiff materials in speaker domes, particularly in the larger units required for large auditoriums, for example. The resonance frequencies of such larger units cannot easily be displaced to high frequencies beyond the point at which they impact on the audible perception, and the nature of high stiffness materials and high rigidity structures is generally to have low damping or a high Q factor at resonance.

SUMMARY OF THE INVENTION

According to a first aspect of the invention, a rigid three-dimensional component formed of diamond, preferably fabricated to net shape by CVD diamond synthesis, comprises a coating on one or more major surfaces thereof, the coating being designed to enhance the performance and/or to alter the appearance of the component.

In a preferred embodiment of this aspect of the invention, the coating is designed to act as a damping medium. As such, a surface coating provides the optimum location to provide damping of transverse waves propagating on the surface of the structure, and an adequate location to provide damping of compression waves within the plane of the structure.

The damping medium preferably provides significant damping, even in thin film form, whilst providing low additional mass to the component, hereinafter referred to as low additional sheet density.

The coating may not be applied uniformly to the structure, but may for example be thicker in regions where the structure is less sensitive to the mass being added to the structure, particularly if these regions are equally, or in some instances more effective, in providing the benefit of damping obtained from the coating to the component as a whole.

In another preferred embodiment of this aspect of the invention, the coating also, or alternatively, provides aesthetic qualities to the component. For example, in high value applications where the structure is visible, it may be appropriate to use coatings to modify the colour, colour uniformity, or transparency of the component.

The component is preferably a speaker dome.

According to a second aspect of the invention, a rigid three-dimensional speaker component, in particular a speaker dome, is formed of a material of high stiffness, in particular of high specific stiffness, or of a material of high rigidity, such as a partially densified material having high rigidity, and comprises a coating on one or more major surfaces thereof, the coating being designed to enhance the performance of the component and/or to alter the appearance of the component.

Once again, the coating may be provided as a damping medium and/or to provide aesthetic qualities to the component, as described above.

In the case of a speaker dome, the coating may be placed on either the inside surface of the dome or the outside surface of the dome, or a combination thereof. Preferably a coating for aesthetic purposes is placed on the outside or visible surface of the dome, particularly where the dome is formed from diamond.

A particularly useful combination is a coating on the outside or visible surface of the dome to modify or control aesthetics, and a coating, which may be the same or a different coating, on the inside or non-visible surface of the dome to modify or provide damping characteristics.

Suitable coating materials include metals such as Ti, Au and Al, for example, and polymers, plastics and other solid organic materials including polymer based paints, resists and photo-resists, for example.

Metals are particularly useful for aesthetic purposes, with the preferred metals being Ti and Au. They can, however, also provide damping, the preferred metals being Au and

Al. In aesthetic applications, the thickness of the coating can be quite thin, and not add significantly to the overall sheet density of the structure.

Polymers and plastics are particularly good at providing damping, particularly those based on long chain molecules. An important issue here is long term adhesion, but in addition consideration must be given to the sheet density added to the structure and the impact this has on the resonance behaviour, since layers of significant thickness are generally required. By careful selection of a coating material it is possible to provide both aesthetic and damping benefits from the use of a single coating material, which may be applied to one or both major surfaces.

The component of the invention preferably comprises a dome segment having an integral coil mounting flange or tube, such that the component is suitable for use as a speaker dome, with one or more coatings as described above.

In a particularly preferred embodiment of the invention, the component is a high performance tweeter dome, and particularly a high power tweeter dome suitable for high acoustic power projection, such as required in auditoriums and the like.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The invention is directed at the formation of rigid three-dimensional components having relatively low mass, and which are coated to provide additional damping or aesthetic characteristics.

The rigid three-dimensional component is formed of a material of high stiffness, and preferably of high specific stiffness, or of a partially densified material that has high rigidity, and is coated on one or more major surfaces thereof. The coating is designed to enhance the performance and/or to alter the appearance of the component.

The rigid three-dimensional component, which is preferably a speaker component, in particular a speaker dome, onto which the coating is applied may comprise one of:

a) a diamond structure, fabricated to net shape by CVD diamond synthesis;

b) a densified metal or metal alloy matrix composite embedded with ultra-hard particles or grit, preferably diamond and/or cBN particles or grit;
c) a partially densified metal or metal alloy matrix composite embedded with ultra-hard particles or grit, preferably diamond and/or cBN particles or grit; or
d) a partially densified metal or metal alloy

For clarity, certain of the terminology is defined below.

Stiffness is a specific technical term relating to the Elastic Modulus (Young's Modulus) of a material:

Stiffness = Young's Modulus = E.

Often a second key parameter is the density of a material, and so a further term is defined as:

Specific Stiffness = E / ρ, where ρ = density.

However, using material with the same stiffness it is possible to construct structures which are much less compliant than others, for example comparing I beams over flat plates. Thus:

Rigidity = structures resistance to deformation by bending.

In a structural foam or partially densified material, or in a structure comprising dissimilar layers such as a diamond dome coated with other materials a further key parameter is the sheet density or density per unit area of the sheet:

Sheet density = ρ/A, where A = area in the plane of the sheet.

In a dome or similar three-dimensional structure, the rigidity is a function of the wall or shell thickness of the dome, and also parameters such as the radius of the sphere of which the dome forms a part and the proportion of the sphere which forms the dome.

These definitions of stiffness, specific stiffness, rigidity and sheet density are assumed throughout this specification.

With reference to a three-dimensional component or body formed from diamond or densified metal or metal alloy matrix composite embedded with ultra-hard particles or grit, preferably diamond and/or cBN particles or grit, onto which coatings are applied in order to modify the damping or resonant behaviour of the structure, the coating or coatings and the body onto which they are applied will preferably fulfil one or more of the following criteria:

a) the body will be formed from a thin layer, and in particular the thickness of the layer forming the body will preferably not exceed 500 μm, and more preferably not exceed 200 μm, and even more preferably not exceed 100 μm, and even more preferably not exceed 70 μm, and most preferably not exceed 50 μm;

b) the thickness of the layer forming the body will preferably exceed 5 μm, and more preferably exceed 10 μm, and even more preferably exceed 20 μm, and even more preferably exceed 30 μm, and most preferably exceed 40 μm;

c) the coating providing modification of the damping or resonant properties may be placed on one or both major surfaces of the structure. Where the coating is placed on one surface only this is preferably the inside surface or the surface which is less visible in normal use;

d) the coating preferably increases the sheet density of the structure by less than 20%, and more preferably by less than 10%, and even more preferably by less than 5%, and even more preferably by less than 2 %, and most preferably by less than 1%.

e) the coating may be fully densified, or it may be only partially densified, or it may be porous. In particular the layer may be foamed;

f) the coating may be uniform in thickness and/or in sheet density across the structure, or its thickness and/or sheet density may vary to optimise the overall damping efficiency whilst minimising the impact on the total sheet density and the break-up or other resonant frequency of the structure;

g) the coating is preferably organic. A particularly advantageous form of coating comprises long organic chains in which the degree of cross-linking can be modified, for example by UV curing, as part of the optimisation of the properties of the layer and in particular its damping efficiency at the frequencies of interest;

h) the organic layer preferably contains heavy elements such as chlorine, bromine etc. in halo-organic structures, or it may contain metal atoms.

i) the coating itself may comprise more than one layer, the first layer for example providing a good adhesion to the surface of the rigid structure, and the second layer providing the damping efficiency required.

In addition, the coating must be adherent for the expected life of the product, and retain its mechanical/damping properties without substantial change over that product life, and under the normal environmental conditions applicable to the product.

With reference to a three-dimensional component or body formed from diamond or densified metal or metal alloy matrix composite embedded with ultra-hard particles or grit, preferably diamond and/or cBN particles or grit, onto which coatings are applied in order to modify or enhance the aesthetic properties of the structure, the coating or coatings and the body onto which they are applied will preferably fulfil one or more of the following criteria:

a) the body will be formed from a thin layer, and in particular the thickness of the layer forming the body will preferably not exceed 500 µm, and more preferably not exceed 200 µm, and even more preferably not exceed 100 µm, and even more preferably not exceed 70 µm, and most preferably not exceed 50 µm;

b) the thickness of the layer forming the body will preferably exceed 5 µm, and more preferably exceed 10 µm, and even more preferably exceed 20 µm, and even more preferably exceed 30 µm, and most preferably exceed 40 µm;

c) the coating providing modification or enhancement of the aesthetic properties of the structure may be placed on one or both major surfaces of the structure.

Where the coating is placed on one surface only this is preferably the outside surface or the surface which is more visible in normal use. Except in applications where both surfaces are visible, the coating would preferably be applied to one surface only;

d) the coating preferably increases the sheet density of the structure by less than 3%, and more preferably by less than 1%, and even more preferably by less than 0.5%, and even more preferably by less than 0.2 %, and most preferably by less than 0.1%;

e) the coating is preferably fully dense, or as fully dense as the method of application practically allows;

f) the coating may vary in thickness across the structure, but in general is uniform in thickness, at least to the degree allowed by the methods of applying the coating, in those regions where it is present. The coating may be deliberately patterned to provide additional visual impact or another visible characteristic, said patterning comprising windows in the coating or combination of regions of different metals or other materials in order to form a visible pattern;

g) the coating is preferably metal or metal alloy;

h) the coating itself may comprise more than one layer, the first layer for example providing a good adhesion to the diamond surface, for which Ti is particularly applicable, and the second layer providing the optical opacity and other characteristics such as colour required.

Where desired, the aesthetic coating may provide for the marking of the rigid structure with a trademark or other character or symbol. This symbol can be provided as a variation in colour between regions, for example using a Ti background and Au characters, or by leaving transparent apertures in the coating. The latter is particularly applicable with diamond structures, for example diamond speaker domes, since the dome can then have a backlight and the character made visible as an illuminated region of the dome.

In particular the invention relates to the use of such components in the application of loudspeaker drive units.

The component fabricated by any of the above means may be a dome segment, which may have an integral coil mounting flange or tube so that it is suitable for use as a speaker dome. In particular, the component is a high performance tweeter dome. Preferably, the tweeter dome demonstrates one or more of the following properties in combination, when tested in an ideal mount essentially free of effects from the surround:

a) a break-up frequency (BUF), for a speaker dome of radius of curvature 20 mm and segment diameter of 26 mm, scaled appropriately for other sizes, that is greater than 31 kHz, preferably greater than 45 kHz, more preferably greater than 55 kHz, even more preferably greater than 65 kHz, and most preferably greater than 75 kHz;

b) a deviation in the on axis response curve from the modelled ideal on axis response curve, allowing for phase roll-off, measured at 3/9 BUF, preferably at 4/9 BUF, more preferably at 5/9 BUF kHz, and more preferably at 6/9 BUF, and most preferably 7/9 BUF, which is less than 5 dB, preferably less than 3 dB, more preferably less than 2 dB, even more preferably less than 1, and most preferably less than 0.5 dB; and

c) a deviation in the on axis response curve from a flat response measured at 3/9 BUF, preferably at 4/9 BUF, more preferably at 5/9 BUF kHz, and more preferably at 6/9 BUF, and most preferably 7/9 BUF, which is less than 5 dB, preferably less than 3 dB, more preferably less than 2 dB, even more preferably less than 1, and most preferably less than 0.5 dB.

A tweeter to the above specification can be used to provide output to modern audio sources with higher audio quality and improved aesthetics over alternative solutions.

In a preferred version of this embodiment of the invention, the high performance tweeter dome is fabricated to one or more of the following criteria:

a) the tweeter is based on a dome which is convex when viewed from the side of the listener;

b) the tweeter dome is axially symmetric and based on a parabola in which the two axes a, b (where a>=b) are such that a/b is less than 1.5, preferably less than 1.2, more preferably less than 1.1, even more preferably less than 1.05, and most preferably less than 1.01;

c) the tweeter dome is fabricated with an integral axial tube component that either directly provides the former for the voice coils or alternatively provides the means of mechanical attachment for a separate voice coil former, made for example from Al or Kapton;

d) the diameter of the domed area of the tweeter exceeds 24 mm, preferably exceeds 35 mm, more preferably exceeds 45 mm, even more preferably exceeds 55 mm, and most preferably exceeds 65 mm, and the radius of curvature of the tweeter dome exceeds 18 mm, preferably exceeds 26 mm, more preferably exceeds 33 mm, even more preferably exceeds 40 mm, and most preferably exceeds 47 mm.

The speaker dome of this invention has a number of benefits. Whereas diamond, the material with the highest known specific stiffness, can be used to fabricate speaker domes less than 30 mm in diameter where the first break-up frequency is at or at least near 70 kHz, removing any significant effect on the audible frequencies up to 20 kHz, larger diameter tweaters which are generally required for higher power output as may be used in auditoriums etc, also require a larger radius of curvature, and both these characteristics reduces the break-up frequency of the dome. The low damping behaviour of diamond then becomes a disadvantage. However, by combining a very high specific stiffness material such as diamond, or a very rigid structural design such as a partially densified diamond metal matrix composite, with a surface coating which provides suitable damping without substantially affecting the sheet density and thus the break-up frequency, the overall acoustic performance of the speaker can be improved.

Viewing the composite structure, in order to obtain the most efficient use of the high damping efficiency layer, locating the damping layer at an external surface is the ideal location to damp out transverse waves, since the deformation is maximised at this point. Transverse waves are the main source of acoustic interference, and or the main type of wave excited by the oscillation of the dome perpendicular to its span. Compression waves in the plane of the dome are equally damped by positioning the damping layer anywhere through the thickness of the composite structure, and so location at the surface is satisfactory, although compression waves are not considered to be a major cause of acoustic interference.

Coatings with high damping efficiency can be applied by a number of techniques, including:

a) application as an organic in a solvent medium, by spinning, spraying or coating, using similar techniques to paints or resists;

b) application as a multicomponent system which sets by chemical reaction, in much the same way as an epoxy resin;

c) application as a single component system which is cured or set by thermal, optical or other means, such means including oxidation in contact with air, baking, UV curing etc.

In each case above, the coating may then be modified further by baking, UV curing etc. in order to obtain the precise damping efficiency required.

Coatings for aesthetic applications can also be applied by a number of techniques, including: sputtering coating, evaporation techniques, CVD coating techniques, plasma spraying, and thermal spraying. In addition, a range of organic chemistry based techniques such as sol-gel processing can be used.